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CANDICIDIN, A NEW ANTIFUNGAL ANTIBIOTIC[1]

HUBERT LECHEVALIER, ROBERT F. ACKER, CHARLES T. CORKE, CONRAD M. HAENSELER AND SELMAN A. WAKSMAN

(WITH 4 FIGURES)

The rapid development of our knowledge of chemotherapeutic agents of microbial origin, known as antibiotics, has yielded in recent years a number of substances which have made possible the control of most bacterial and rickettsial infections. Relatively little progress has been made, however, in the control of virus and fungal diseases. These still remain a challenge to investigators in the field of antibiotics. The need for effective antifungal agents has been felt more strongly lately since a certain number of patients receiving antibiotics by the oral route of administration, for the control of bacterial infections, became more susceptible to secondary infections by fungi, notably yeast-like organisms (**6**). The potential importance of antibiotics active against phytopathogenic fungi may also be mentioned, even though such antibiotics have so far played only an insignificant role in the control of plant diseases (**1**).

ISOLATION OF CANDICIDIN-PRODUCING ORGANISM

A group of 197 cultures of actinomycetes belonging to the genus *Streptomyces*, isolated from soils and manures, were tested for their

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University—the State University of New Jersey, Departments of Microbiology and Plant Pathology.

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activity against *Ceratostomella ulmi*, the causative agent of Dutch elm disease. The cultures were streaked on plates containing potato or peptone-glucose agar media, and incubated for 2 to 4 days at 28° C. The plates were then cross-streaked with a fast-growing culture of *C. ulmi* (Pomerleau's strain), and further incubated at the same temperature for 4 to 5 days. The zone of inhibition of growth of *C. ulmi* was measured. The initial tests indicated that nearly 50 per cent of the isolates were antagonistic, in varying degrees, against the test organism.

Seven cultures, selected on the basis of their high consistent activity by the cross-streak method, were used for further study in liquid media. They were grown, in shake flasks, using nutrient and yeast glucose broths; in these media, four of the cultures showed no activity against bacteria (*Escherichia coli, Staphylococcus aureus*) or fungi (*C. ulmi, Aspergillus niger*). Two cultures exhibited both antibacterial and antifungal activity. Their antimicrobial spectra strongly indicated, however, that they were neomycin- and fradicin-producing strains, although culturally they were different from *Streptomyces fradiae* (**3**). The last of the seven active isolates yielded broths which possessed strong antifungal activity; this culture was, therefore, selected for further study. It was found to produce a new antibiotic substance which had no antibacterial activity but was characterized by marked fungistatic and fungicidal properties, especially against *Candida albicans*. It was designated, therefore, as *candicidin*.[1a]

CHARACTERIZATION OF CANDICIDIN-PRODUCING ORGANISM

The candicidin-producing culture was isolated from a sample of cow manure during the summer of 1948. It belongs to the *Streptomyces griseus* group, although it showed certain minor differences from the typical streptomycin-producing strains of this organism (**5**). It has been deposited in the culture collection of the Department of Microbiology of Rutgers University as No. *3570*.

A study was made of the comparative cultural and morphological properties of a typical *S. griseus* and of No. *3570* culture. The

[1a] Preliminary data concerning this new antibiotic were presented at the Annual Meeting of The Mycological Society of America. Ithaca, September 10, 1952.

TABLE I

MORPHOLOGICAL AND CULTURAL PROPERTIES OF *S. griseus* AND NO. *3570*

	S. griseus	No. *3570*
Structure of aerial mycelium on yeast-dextrose and Czapek's agars	Branching filaments forming tufts of straight sporulating hyphae.	Branching filaments forming tufts of curved sporulating hyphae.
Gelatin stab	Rapid liquefaction. Greenish-yellow or cream colored surface growth with brownish tinge.	Rapid liquefaction. Flaky growth falls to bottom. Faint brownish pigment.
Czapek's agar	Thin, colorless, spreading, becoming olive buff. Aerial mycelium thick powdery, water green.	Poor growth, thin, cream colored, with brownish dark tinge in vegetative growth and greenish tinge in aerial mycelium.
Yeast dextrose agar	Cream colored growth elevated in center, gray-green powdery aerial mycelium.	Cream colored lichnoid growth, grey powdery aerial mycelium.
Nutrient agar	Abundant cream colored, lichnoid growth powdery, white to light grey aerial mycelium.	Cream colored growth thin, powdery, light grey aerial mycelium.
Glucose-asparagine	Thin cream colored growth. Light grey aerial mycelium. No soluble pigment.	Same.
Litmus milk	Cream colored ring. Coagulation with rapid peptonization, becoming alkaline.	Coagulation rapid with peptonization, becoming alkaline.
Potato plug	Brownish, lichnoid growth, covered with white powdery aerial mycelium. No soluble pigment.	Same.
Pigment	Not soluble in medium	Same.
Nitrate reduction	+	+
Optimum temperature	37° C	37° C
Sensitivity to phage M-1	Sensitive	Resistant
Sensitivity to streptomycin	100 mcg/ml	2 mcg/ml
Source	Garden soil	Cow manure

results are summarized in TABLE I. Culture *3570* forms tufts of sporulating hyphae characteristic of *S. griseus;* they differ from the typical forms only in that the aerial hyphae are slightly curved instead of being straight. Minor differences were also found in some of the cultural properties, such as a relatively poor growth of No. *3570* on Czapek's agar. These differences from the type species are no greater than those already reported (**5**) for different strains of *S. griseus.*

ASSAY AND STANDARIZATION OF CANDICIDIN

Candicidin did not diffuse readily in agar media; hence dilution assays were employed rather than the common diffusion methods. For routine procedures, the streak dilution method (**4**) was used. When there was a possibility of bacterial contamination, neomycin, in a concentration of 8 mcg per ml of medium,[2] was added. The same medium in liquid form was used for turbidimetric assays.

One candicidin unit was defined as the minimum amount of antibiotic per one ml of peptone-glucose agar which completely inhibited the growth of *Candida albicans 204.* An 18-hour-old culture of *C. albicans* grown on yeast-glucose agar was used as the inoculum.

The age of the *C. albicans* culture used as inoculum in the agar streak assay was found to cause no appreciable variation. No change in assay results was observed when the glucose concentration in the test agar was varied from 0.25 to 2.5%. The pH and the sodium chloride concentration of the medium were found, however, to have a marked effect on the value of the results obtained. The greater the pH and the sodium chloride concentration, within certain limits, the higher were the assay values obtained.

PRODUCTION OF CANDICIDIN

Candicidin was produced by growing culture No. *3570* on yeast-glucose medium,[3] at 28° C. in shake cultures, using 2000 ml Erlenmeyer flasks. A primary inoculum was prepared by transferring

[2] The medium consisted of 1% glucose, 0.5% peptone, 0.5% NaCl, 0.3% meat extract, 1.5% agar, in tap water. The pH was adjusted to give 7.2 after sterilization.

[3] Bacto yeast extract 1%, cerelose (commercial glucose) 1%, and tap water; pH adjusted before sterilization to 7.5 with 20% NaOH.

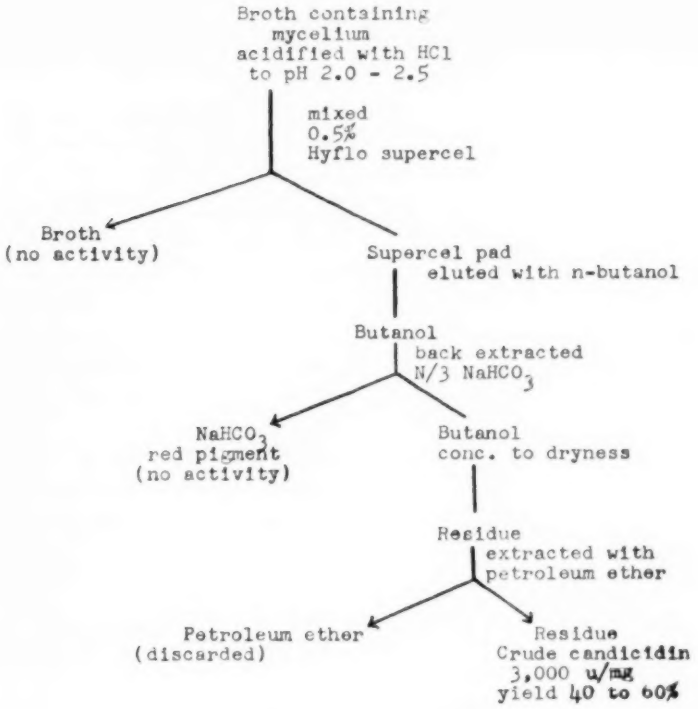

FIG. 1. Extraction of crude candicidin.

spores from agar slants into 250 ml Erlenmeyer flasks containing 100 ml medium. These flasks were shaken for 24 hours and the contents used to inoculate a series of similar flasks (secondary inoculum), which were then incubated for another 24 hours. The large Erlenmeyers, containing 300 ml of medium, were each inoculated with one of the cultures of the secondary inoculum and shaken at 28° C for 4 days. They yielded broths having candicidin potencies of 1000 to 5000 units per ml. Broths having potencies of 3000 to 5000 units were also obtained on a glutamic acid-glucose medium [4] in 250 ml flasks containing 100 ml of medium inoculated

[4] K_2HPO_4—0.5 gm, $MgSO_4 \cdot 7H_2O$—0.2 gm, $Fe(SO_4)_3 \cdot 9H_2O$—0.1 gm, $ZnSO_4 \cdot 7H_2O$—0.01 gm, anhydrous glucose—10 gms, L-glutamic acid—10 gms, distilled water 1000 ml; pH adjusted to 7.3 before autoclaving at 15 lbs for 15 minutes.

with a washed one-day-old yeast-glucose shake culture and incubated on a shaking machine, at 28° C, for 5 days.

EXTRACTION AND PURIFICATION OF CANDICIDIN

As a result of growth of the organism under the conditions outlined above, the culture attained a pH value of 7.5 to 8.5. The first candicidin preparations were made by extraction of the broth with n-butanol at pH 8.0. The butanol was concentrated to dryness and the residual solid suspended in water and freeze dried. The solid preparations obtained by this method had a potency of 900 units per mg.

A method of extraction giving materials having approximately three times this activity was later developed. The whole culture,

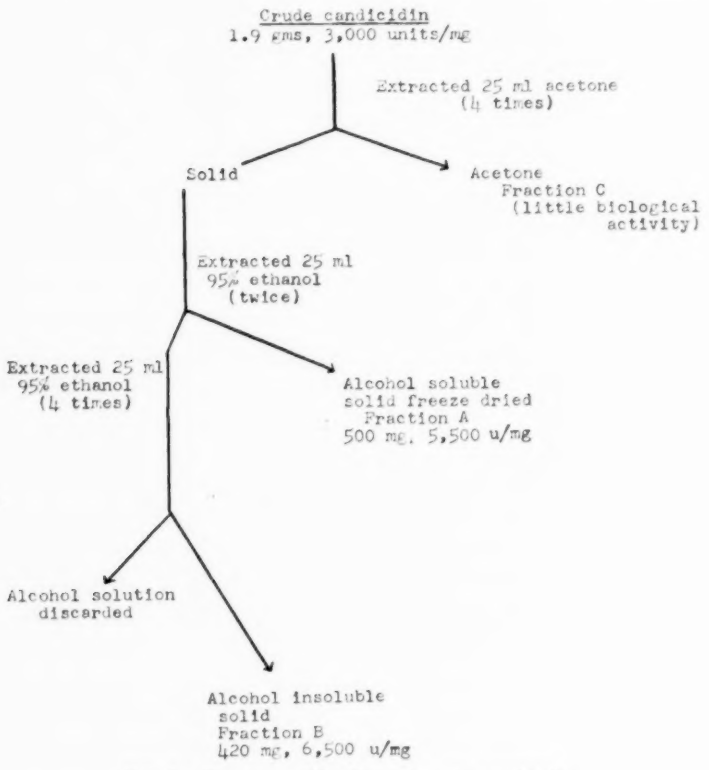

FIG. 2. Further purification of crude candicidin.

TABLE II

ANTIFUNGAL SPECTRA OF THREE FRACTIONS OBTAINED FROM CRUDE CANDICIDIN

Assay on peptone glucose medium. Cultures incubated, at 28° C, for 48 hours

	Dilution units per mg.		
	Fractions		
	A	B	C
Candida albicans	2,000	3,000	200
Ceratostomella ulmi	>10,000	>10,000	250
Trichoderma sp.	90	250	<30
Penicillium notatum	<90	<250	<30
Aspergillus niger	200	200	<30
Rhizopus nigricans	<90	<250	<30

including the mycelium, was treated with HCl to give a pH of 2.5, and stirred 10 minutes with 0.5 per cent Hyflo-supercel. The supercel pad, which was filtered off, retained the antibiotic present both in the broth and in the mycelium. An elution was made by mixing equal volumes of supercel and n-butanol. The solvent, which was deep red and contained the active substance, was filtered off. The pigment was removed by extracting the butanol exhaustively with 2 per cent of its volume of N/3 $NaHCO_3$. The residual butanol was concentrated to dryness *in vacuo* and extracted with petroleum ether. The active substance was suspended in water and freeze-dried (FIG. 1). The preparation obtained by this method was designated as "crude candicidin."

On further purification by the use of organic solvents, the crude candicidin yielded three fractions (FIG. 2). Two of these (A and B) had almost equivalent biological activity and could not be differentiated by their antimicrobial spectra (TABLE II); the third fraction (C) had negligible activity. The ultraviolet absorption spectra of the three fractions were found to be very similar, however (FIG. 3). When compared by paper chromatography,[5] fractions A and B gave Rf values varying from 0.4 to 8.0 (Fig. 4) and

[5] Method: ascending chromatography. System: 0.2% acetic acid in butanol saturated with water. Paper: Whatman No. 4. Development: 18 hours, at 23° C. Paper strips, after evaporation of solvent, plated out on assay medium seeded with an 18-hour-old culture of *C. albicans*. Sterile zones read after 18 hours of incubation at 37° C.

fraction C had an Rf value of 0.95. Fraction C diffused readily in the assay medium but had only a reducing effect on the growth of *C. albicans,* whereas fractions A and B did not diffuse from the paper but gave a sterile zone under the paper strip (FIG. 4).

PURIFICATION BY CHROMATOGRAPHY

Crude candicidin could also be purified by separation on a chromatographic column composed of cellulose powder. The column

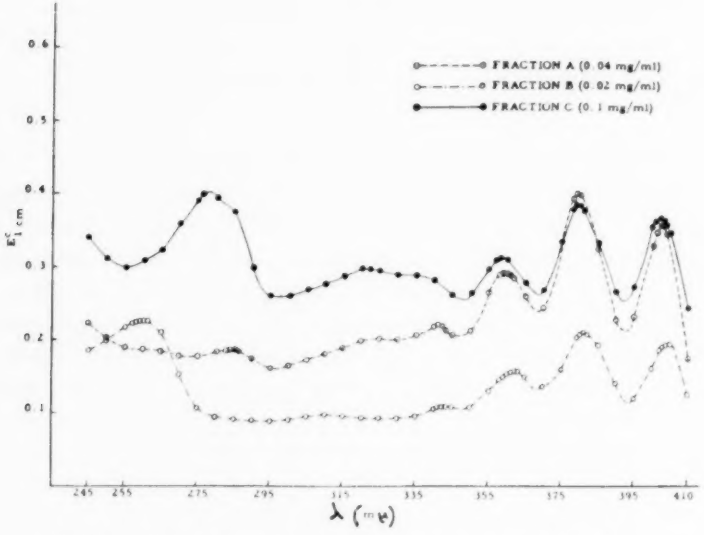

FIG. 3. Ultra-violet absorption spectra of candicidin fractions.

was prepared by pouring a water suspension of cellulose powder into a glass tube; while still moist, it was washed successively with equal amounts of ethylene glycol monomethyl ether, ethanol, and chloroform. The column was then loaded with a chloroform suspension of crude candicidin. The first elution was carried out with 95 per cent ethanol and yielded a reddish brown eluate, fraction C. Further elution was accomplished by the addition of an equal mixture of ethylene glycol monomethyl ether and ethanol. This mixture eluted first a reddish brown compound, fraction A, followed by a greenish compound, fraction B (TABLE III). So

far as could be determined from the comparison of their biological spectra, solubility properties, ultraviolet adsorption spectra, and Rf value upon paper chromatography, these three fractions A, B, and C, were identical with the corresponding fractions obtained by solvent extraction.

TABLE III

CHROMATOGRAPHY OF CRUDE CANDICIDIN ON CELLULOSE POWDER*

Column loaded with a chloroform suspension of crude candicidin (40,000,000 units), column size 2.5 × 70 cm

Solvent cut no.	Color	Solvent†	Volume ml	Assay units/ml	Fraction
1	yellow	chl	250	500	
2	yellow red	chl	10		
3	brown red	chl + EOH	25	4,000	
4	brown red	EOH	45		C
5	orange	EOH	30	2,500	
6	light orange	EOH	27		
7	yellow	EOH	95	500	
8	yellow	EOH	83	800	
9	brown red	EOH + EGMME	30	70,000	
10	brown	EOH + EGMME	90	70,000	A
11	green brown	EOH + EGMME	50	60,000	
12	green brown	EOH + EGMME	150	40,000	
13	green brown	EOH + EGMME	35	8,000	B
14	green brown	EOH + EGMME	70	8,000	
15	green yellow	EOH + EGMME	200	4,000	
16	green yellow	EOH + EGMME	200	1,000	
17	green yellow	EOH + EGMME	215	700	
18	green yellow	EOH + EGMME	300		

Total recovery (all factors) about 50%.

* Whatman ashless powder, chemically prepared, standard grade.
† Chl = chloroform
EOH = ethanol
EGMME = ethylene glycol monomethyl ether

ANTIMICROBIAL ACTIVITY OF CANDICIDIN

The antifungal activity of crude candicidin was assumed to be due to the antibiotic action of fractions A and B, since fraction C exhibited little or no activity. At the same time, the antibiotic spectra of A and B were found to differ only in degree of inhibition of the test organisms (TABLE II).

The spectrum of crude candicidin (TABLE IV) indicates no activity against bacteria, mycobacteria, or actinomycetes. This preparation was markedly active against yeasts and yeast-like fungi

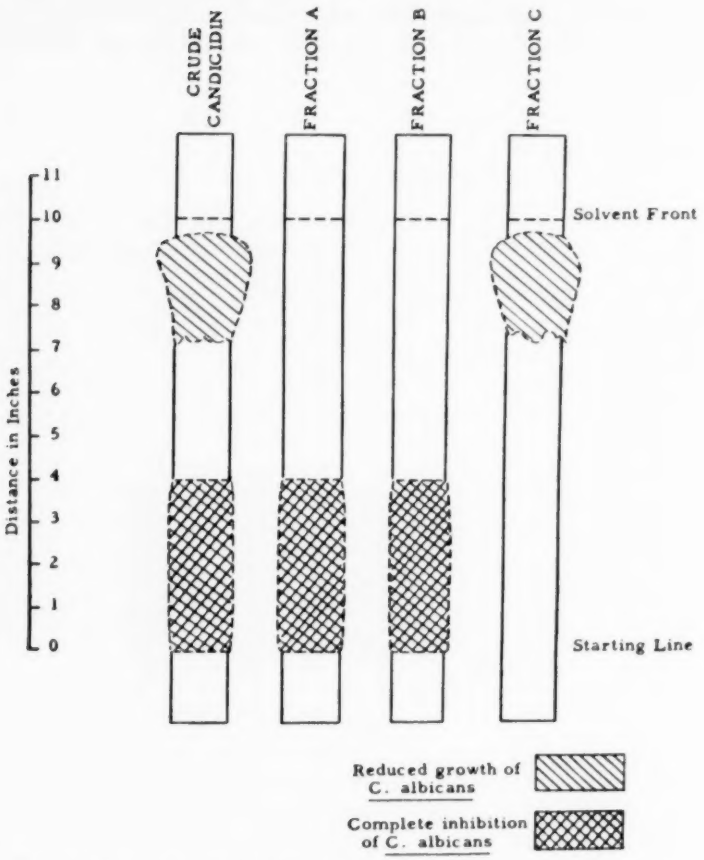

FIG. 4. Paper chromatography of candicidin fractions. Action of the developed paper strips on the growth of *C. albicans*.

(TABLE V). TABLE VI shows the antifungal activity of fraction B.

The fungicidal activity of candicidin was determined by mixing various amounts of crude material with a heavy suspension of *C. albicans* cells and allowing the mixture to stand at room temperature. Samples were taken at regular intervals and streaked on peptone-glucose agar to determine viability of the cells. Apparent sterilization of a suspension of resting cells was effected in 3 hours with a concentration of 5 mcg/ml; in the case of growing

TABLE IV

ANTIBACTERIAL SPECTRUM OF CRUDE CANDICIDIN

Streak dilution assay carried out on nutrient agar
Incubation at 37° C for 18–48 hours

Name of organism	Micrograms per ml for complete inhibition
Escherichia coli	>100
Aerobacter aerogenes	>85
Serratia marcescens	>85
Pseudomonas fluorescens	>85
Bodenheimer's organism	>85
Staphylococcus aureus	>100
Bacillus subtilis	>100
Mycobacterium phlei	>85
M. avium	>85
Mycobacterium sp. 607	>85
Streptomyces fradiae	>85

TABLE V

ANTIFUNGAL SPECTRUM OF CRUDE CANDICIDIN

Test on peptone-glucose medium. Incubation for 2 to 5 days at 28° C

	Microgram per ml for complete inhibition
Acrostalagmus sp.	>10
Alternaria sp.	0.6–10
Aspergillus niger	5–70
Candida albicans	0.3–0.5
Cercospora kikuchii	8–15
Ceratostomella ulmi (P strain)	0.15–0.3
C. ulmi (H strain)	3–5
Botrytis sp.	16–50
Diaporthe sp.	13–70
Epicoccum sp.	30–50
Helminthosporium sp.	13–20
Isaria sp.	>100
Fusarium sp.	66–>100
Hormodendron sp.	30–50
Oospora sp.	>100
Mucor sp.	3–10
Penicillium notatum	5–100
Pestalozzia sp.	0.6
Phoma sp.	66–100
Polyporus sulphureus	8–20
Pullularia sp.	1–3
Pythium sp.	66–100
Saccharomyces cerevisiae	<0.15–0.3
Sclerotium rolfsii	>100
Spicaria sp.	>100
Stemphylium sp.	>85
Stysanus sp.	>100
Trichoderma sp.	20–30
Ustilago zeae	1–20
Verticillium sp.	0.6–>100

TABLE VI

ANTIFUNGAL SPECTRUM OF FRACTION B

Test run on peptone-glucose agar
* incubated at 28° C
† incubated at 37° C
Results in micrograms per ml for complete inhibition

Organism	Days of incubation 1	5
* *Aspergillus niger*	2	>10
† *Candida albicans*	0.22	1.1
† *C. brumptii*	3.3	>10
† *C. kruseii*	1	2.2
† *C. neoformans*		0.5
† *C. pseudotropicalis*	1.7	>10
† *C. stellatoides*	0.41	1.1
† *C. tropicalis*	2	>10
* *Ceratostomella ulmi* (P strain)		1.1
† *Cryptococcus neoformans*		0.5
* *Fusarium* sp.		10
* *Hormodendrum pedrosoi*		>10
* *Mucor* sp.	0.1	>10
* *Penicillium notatum*		1.1
* *Phialophora verrucosa*		>10
* *Rhizopus nigricans*	>10	>10
* *Saccharomyces cerevisiae*	0.06	0.14
* *Sporotrichum schenkii*		>10
* *Trichophyton gypsum*		>10
* *T. mentagrophytes*		>10

cells 1 mcg/ml was sufficient to sterilize the culture in 3 hours (TABLE VII). When *C. ulmi* and *A. niger* were tested in a similar manner, little fungicidal activity was observed.

TABLE VII

FUNGICIDAL EFFECT OF CANDICIDIN ON RESTING CELLS OF *Candida albicans*

Cell suspension of *C. albicans* in sterile water, treated with various concentrations of crude candicidin, for varying periods of time, transferred to peptone-glucose agar and incubated at 37° C for 18 hours to determine viability.

mcg/ml	Exposure to candicidin in hours 1	2	3	7	20
Control	++++	++++	++++	++	++
250	−	−	−	−	−
100	−	−	−	−	−
50	−	−	−	−	−
10	−	−	−	−	−
5	+	+	−	−	−
1	++	++	+	+	±
0.5	+++	+++	++	++	+
0.1	++++	+++	+++	++	+

PHYSICAL AND CHEMICAL PROPERTIES OF CANDICIDIN

Crude candicidin was soluble in the higher alcohols, but insoluble in benzene, petroleum ether, carbon tetrachloride, xylene, carbon disulfide, ethylene dichloride, ether, or ethyl acetate. Only fraction A was soluble in water (TABLE VIII).

TABLE VIII

SOLUBILITY OF 3 CANDICIDIN FRACTIONS

	Crude candicidin	Fraction		
		A	B	C
Water	+−	+	−	−
Ethanol	+−	+	−	+
Butanol	+	+	+*	+
Glycerol	+			
Benzyl alcohol	+			
Ethylene glycol (EG)	+	+	+*	+
EG monomethyl ether	+	+	+*	+
Chloroform	+−			
Acetone	+−	−	−	+

* Solubility of fraction B solid preparations in these solvents about 100 mcg/ml.
+ = soluble
− = insoluble
+− = partially soluble

Concentrated solutions of candicidin were more stable than dilute solutions. At neutrality, fractions A and B withstood heating for 10 minutes at 60° C and could be kept for a week at 4° C without appreciable loss of activity. TABLE IX illustrates the stability of solutions of candicidin fractions A, B, and C at room temperature.

TABLE IX

STABILITY OF CANDICIDIN SOLUTIONS AT ROOM TEMPERATURE
Candicidin units per ml

Fraction	Hours at 27° C				
	0	3	9	24	168
A	6000	4500	2500	1000	900
B*	6000	4500	2000	1500	1500
C	250	250	200	100	200

Solutions in ethylene glycol monomethyl ether containing 1 mg/ml.
* B solution filtered off on paper.

The three fractions of candicidin had very similar U. V. absorption spectra (FIG. 3), which suggests a close chemical relationship between these fractions. The following peaks of maximum absorption were recorded:

Fraction A: 360, 380, 403 mμ
Fraction B: 362, 381, 404 mμ
Fraction C: 358, 379, 402 mμ

Elementary analysis of fraction A gave the following values on an ash-free basis: H = 9.6%, C = 62.9% and N = 4.7%. Fraction B was somewhat different: H = 9.9%, C = 57.8% and N = 7.3%. Some sulfur could also be detected in both fractions but it is very possible that it was present as an impurity in the ash.

Crude candicidin did not dialyze through a cellophane membrane.

TOXICITY OF CANDICIDIN TO EXPERIMENTAL ANIMALS

Acute toxicity testing of various candicidin preparations, using twenty gram albino mice, gave the following LD_{50} by the subcutaneous route of injection: crude candicidin 663 mg/kg, fraction A 277 mg/kg, fraction B 159 mg/kg. Intraperitoneally the following LD_{50} were obtained: crude candicidin 79 mg/kg, fraction A 47 mg/kg, fraction B 53 mg/kg. Necrosis was noted at the site of inoculation when the concentration of crude candicidin reached 0.63 mg per 20 gm mouse. Necrosis was also observed when 0.16 mg or more of fraction A or 0.32 mg or more of fraction B was injected per mouse.

EFFECT OF CANDICIDIN ON PLANT GROWTH

Crude candicidin was found to have no ill effect upon the germination of pea seeds in concentrations of 125 mcg/ml or less. Two ml of the candicidin solution were added to 5.5 cm petri dishes containing 5 to 10 pea seeds. These dishes were placed inside regular size (9.0 cm) dishes containing an absorbent pad saturated with water. The seeds were incubated 5 to 6 days at 20° C (TABLE X).

Spraying young bean plants once a week with an aqueous suspension of crude candicidin (660 mcg/ml) resulted in a decrease

TABLE X

EFFECT OF CRUDE CANDICIDIN ON GERMINATION OF PEA SEEDS

	Candicidin	Germination	Root growth, in per cent of control
	mcg/per plate	*per cent*	
No pretreatment	†1000	40	43
	†500	100	54
	250	100	120
	100	100	100
	50	100	103
Seeds soaked in water 24 hours prior to testing with candicidin	†1000*	100	90
	†500*	100	90
	250	100	103
	100	100	114
	50	100	103

* Development of primary leaves inhibited at these concentrations.
† With other batches of peas, no reduction of the root length and no inhibition of the primary leaves were observed at these concentrations.

of a powdery mildew infection. The spraying was started about 5 days before the mildew infection was apparent on the plant leaves. One single spraying before the appearance of the mildew did not show any therapeutic value. No toxic reactions were noted.

COMPARISON OF CANDICIDIN WITH OTHER ANTIFUNGAL ANTIBIOTICS PRODUCED BY ACTINOMYCETES

Candicidin was compared with other antibiotics produced by actinomycetes which were antifungal and lacked antibacterial activity. The ultraviolet absorption spectra of the candicidin fractions were distinctly different from the spectra for actidione, antimycin A, fradicin, fungicidin, and rimocidin. No direct comparison was made between candicidin and actinone, but a comparison of the data of Ikeda *et al.* (2) with our own showed that actinone was less toxic and less active *in vitro* than was candicidin. The solubility properties and the nitrogen content of the two substances were also different.

ACKNOWLEDGMENT

The authors are greatly indebted to Dr. W. Ruigh, formerly of the Department of Microbiology of Rutgers University, who devised part of the method of extraction of crude candicidin reported

in this paper and to Dr. V. Groupé and Miss L. Pugh, who determined the acute toxicity of the various preparations. The advice of Dr. Robert L. Peck and Dr. K. Folkers of Merck and Company was most valuable during this investigation. The authors also wish to thank Mr. Edwin Bailey and Mr. Aldrage B. Cooper for their technical assistance.

SUMMARY

A group of 197 cultures of actinomycetes were tested for their antibiotic activity against *Cerastostomella ulmi*. This screening program yielded a strain of *Streptomyces griseus* which produced an antibiotic substance that was very active against yeasts, yeast-like fungi, and *C. ulmi;* it was not very active against filamentous fungi and had no activity against the bacteria tested. The new antibiotic was different from the known antifungal agents actidione, antimycin A, fradicin, fungicidin, rimocidin and actinone.

Because of its marked fungistatic and fungicidal action against *Candida albicans*, the new antibiotic was designated as candicidin. A crude extract of candicidin could be obtained from liquid culture media, by adsorption on Hyflo-supercel and elution with n-butanol. Crude candicidin could be further fractionated, by solvent extraction and chromatography on cellulose powder, into two very active fractions (A, B) and an almost inactive fraction C.

Crude candicidin was fungicidal against growing and resting cells of *Candida albicans* in the concentration of 1 to 5 mcg/ml. It had no injurious effect upon the germination of pea seeds in concentration of 125 mcg/ml. Spraying young bean plants once a week with an aqueous suspension of crude candicidin (660 mcg/ml) resulted in a decrease of a mildew infection.

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PIGMENT PRODUCTION IN CERTAIN OF THE ASPERGILLUS GLAUCUS GROUP[1, 2]

GEORGE THOMAS JOHNSON[3] AND BERNARD S. GOULD

(WITH 8 FIGURES)

Gould and Raistrick (**1934**) have shown that the *Aspergillus glaucus* group, perhaps the most common of the Aspergilli, are characterized by the production of several crystalline pigments: flavoglaucin and auroglaucin (whose structures are given in FIG. 1), as well as a series of substituted anthraquinones and anthranols (Raistrick, **1950**). Flavoglaucin and auroglaucin are substituted

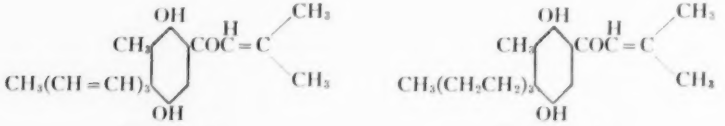

FIG. 1. Structural formulae of the characteristic pigments of the *A. glaucus* group.

toluquinol structures. The present study deals primarily with these latter pigments. Auroglaucin appears to be an oxidation product of flavoglaucin which poses the question as to whether or not these compounds constitute an oxidation-reduction mechanism in the organism. While anthraquinones and anthranols are produced in but relatively small quantity by these molds, flavoglaucin and auroglaucin together can be produced to the extent of 40% or more of the dry weight of the organism. The fact that these pigments perhaps constitute the principal end product of metabolism greatly increases interest in the substances. Flavoglaucin and auro-

[1] This investigation was supported in part by a grant from the A. C. Lawrence Leather Company of Peabody, Massachusetts.

[2] A preliminary report was presented at the Gordon Conference on Microbiological Deterioration, July 2–6, 1951.

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glaucin have been isolated only from members of the *Aspergillus glaucus* group, but compounds of closely related structure have been obtained from other organisms in relatively small yields.[4]

The fact that these pigments constitute so significant a part of the end products of metabolism suggested that a study of the physiology of pigment production might throw some light on the biosynthetic mechanisms involved in the formation of relatively complex molecules from simple carbon sources such as glucose. While Gould and Raistrick (**1934**) in their study of the pigments defined some of the conditions which lead to an increase in pigment production, no attempt to elucidate the mechanism of pigment formation by members of this group has yet been made.

In the *glaucus* group pigment production is extremely good on common carbohydrate sources; further, these Aspergilli are, in general, noted for producing small amounts of ethyl alcohol. Hence it was felt that the problem of pigment biosynthesis might be approached by determining whether disruption of the usually accepted pathways of carbohydrate dissimilation, such as those leading to ethyl alcohol in yeast metabolism, would lead to concomitant interruption of pigment production in species of *Aspergillus*. Results of experiments with the commonly used inhibitors (such as sodium fluoride and iodoacetic acid; cyanide and azide) are reported in the present paper. Studies are also reported on the availability of numerous substances as possible precursors of the pigments. In view of the results obtained during the course of this investigation, particular emphasis was directed toward the possible roles of mannitol, which is so ubiquitous a mold metabolic product, and of glycerol.

MATERIALS AND METHODS[5]

Organism. The organisms used in these studies were *Aspergillus novus* (NRRL Culture No. *46*) and *Aspergillus pseudoglaucus* (NRRL Culture No. *41*), both obtained from Dr. Kenneth

[4] Spinulosin from *Penicillium spinulosum*, *Aspergillus fumigatus*, and *P. cinerascens* (Birkinshaw and Raistrick, **1931**; Anslow and Raistrick, **1938**), fumigatin and 3-hydroxy-4-methoxy toluquinol from *A. fumigatus* (Anslow and Raistrick, **1938**).

[5] We are indebted to Mrs. Ruth Michaels for technical assistance with certain phases of this investigation.

B. Raper of the Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Illinois, to whom we express our thanks.[6] Most of the work to be presented was carried out with *A. pseudoglaucus* since it gave the most consistent growth and was most adaptable to submerged growth. Confirmatory experiments were frequently carried out with *A. novus*. Spore suspensions used for inoculation were made from growths developing on sporulation agar (formulation of Moyer and Coghill, **1946**). The spores produced on each test tube slant were suspended in 15 ml. of sterile water.

Medium. Most of the experiments were carried out in media having the basal salt mixture of Czapek-Dox medium: KH_2PO_4, 1.0 g.; KCl, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; $NaNO_3$, 2.0 g.; water, 1000 ml. The desired carbon source was added as indicated in each experiment reported. Gould and Raistrick (**1934**) found that ammonium salts of organic acids such as ammonium tartrate gave the highest yields of pigment under the growth conditions they had tested. In the present studies, however, such carbon-containing nitrogen sources were avoided since their use would complicate any interpretation of the results obtained. Usually 75 ml. quantities of medium were dispensed in 300 ml. Erlenmeyer flasks and sterilized in an autoclave for 20 minutes at 15 lbs. pressure.

Cultural Conditions. Surface growths. Flasks were inoculated with uniform spore suspensions from sporulation agar using 1 ml. of spore suspension. The flasks were incubated without agitation at $24° \pm 2°$ C. They were exposed to both light and dark.

Submerged cultures. The flasks were inoculated as described above, then placed on a shaking machine where they were continuously agitated. The growths so produced were made up of uniform pellets which remained colorless for about four days and then, over a period of a few days, assumed a uniform brilliant yellow color.

[6] Thom and Raper (**1945**) consider these cultures two strains of a single species (*A. pseudoglaucus*). However the two cultures often present striking biochemical peculiarities and we have distinguished them under the original strain names in the present paper.

Upon extraction the typical flavoglaucin and auroglaucin could be isolated and identified.

Resting preparations from submerged cultures. The submerged pellets constituted a convenient material for studying pigment production, particularly on substrates which did not support good growth. What will be called "resting preparations" were prepared in the following manner. After three or four days growth on the shaker and while the pellets were still colorless, the growths were combined and centrifuged to remove the original growth medium. The collected pellets were then resuspended in sterile solutions of the same composition as the original medium on which they had been grown and washed by centrifugation. This procedure was repeated until the pellets had been washed three times in fresh solutions. Washed "resting preparations" were then resuspended in various substrates, as desired, for determination of the availability of these substrates for growth and pigment production. Aseptic precautions were taken throughout.

Chemical Methods. Estimation of growth. At the end of the desired growth period the mycelium was filtered off, washed thoroughly with cold distilled water, pressed as dry as possible between paper towels, and dried to constant weight at 40° C. This was termed the dry weight.

Extraction of pigments. Total crude pigment. The dried mycelium was mixed with sand, ground to a fine powder in a mortar, and continuously extracted with anhydrous ether in a Soxhlet extractor until no further pigment was removed. The ether extract was evaporated to dryness and the residue was dried in a desiccator to constant weight. This is taken as total crude pigment. A certain amount of fat is included but examination of the extracts indicated that the small amount produced constituted a relatively small percentage of the total crude pigment in all cases.

Fractionation of flavoglaucin and auroglaucin. The ether-extracted crude pigment was refluxed with a small volume of hot pentane or light petroleum (boiling point 50–60° C.). The flavoglaucin can be crystallized from the petroleum extract and the residue which is largely auroglaucin is crystallized from methyl alcohol.

EXPERIMENTAL RESULTS

Effect of concentration of carbon source on growth and pigment production

The concentration of the carbon source markedly influences both growth and pigment production as indicated by the results obtained with surface cultures shown in FIG. 2. Within wide limits, increasing carbon concentration increases both the total growth as well as total pigment. The figure shows the effects of raising the concentration to 20%. Further increases to 50% sugar concentration re-

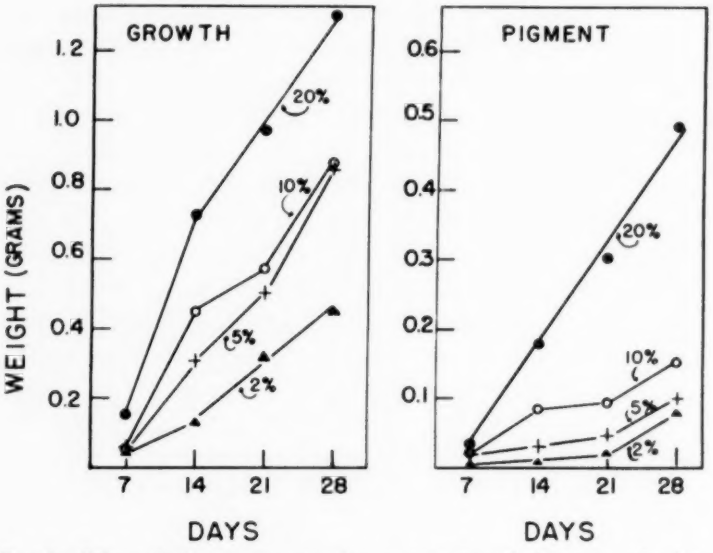

FIG. 2. Effect of glucose concentration on growth and pigment production. *A. pseudoglaucus*. Surface cultures.

sult in further increases of both growth and pigment and the optimum sugar concentration for these processes on glucose medium is above this point. The results shown in FIG. 3 for an analogous experiment with glycerol as the carbon source are strikingly similar.

Availability of various carbon sources for growth and pigment production on surface culture

The results of a typical experiment where surface growths developed on media containing 5% of the desired carbon source are illus-

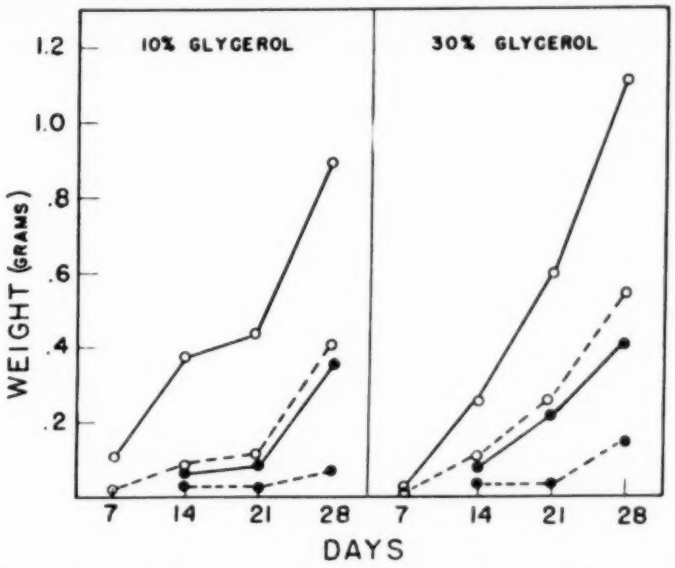

FIG. 3. Effect of glycerol concentration on growth and pigment production. *A. pseudoglaucus*. Surface cultures.
-○———○- = Growth; -○- - -○- = Total pigment;
-●———●- = Pentane; -●- - -●- = Ether fraction.

trated in TABLE I. After 21 days' incubation all of the carbon compounds studied yielded significant growth. Considerable variation in both total growth and pigment production occurred. As a result the amount of pigment per gram of dry mycelium was taken as the

TABLE I

GROWTH AND PIGMENT PRODUCTION BY ASPERGILLUS PSEUDOGLAUCUS ON VARIOUS CARBON SOURCES (SURFACE GROWTHS)

Carbon source*	Total growth g. per flask (dry weight)	Pigment per gram of mycelial growth (dry wt. basis)
Glucose	1.050	.187
Mannose	1.290	.265
Galactose	.913	.243
Fructose	.942	.123
Xylose	1.190	.346
Glycerol	.707	.389
Mannitol	.563	.320
Sorbitol	.781	.055

* Czapek-Dox medium with 5% of carbon source. Temperature 24° C. Grown for 21 days.

most revealing index of the relationship between a carbon source and pigment production. An examination of such values described in TABLE I indicates that glycerol, mannitol, and xylose yielded almost twice the amount of pigment per gram of mycelium as did glucose. Other experiments, one of which is presented in FIG. 4, also suggest a probable preferential role for compounds such as

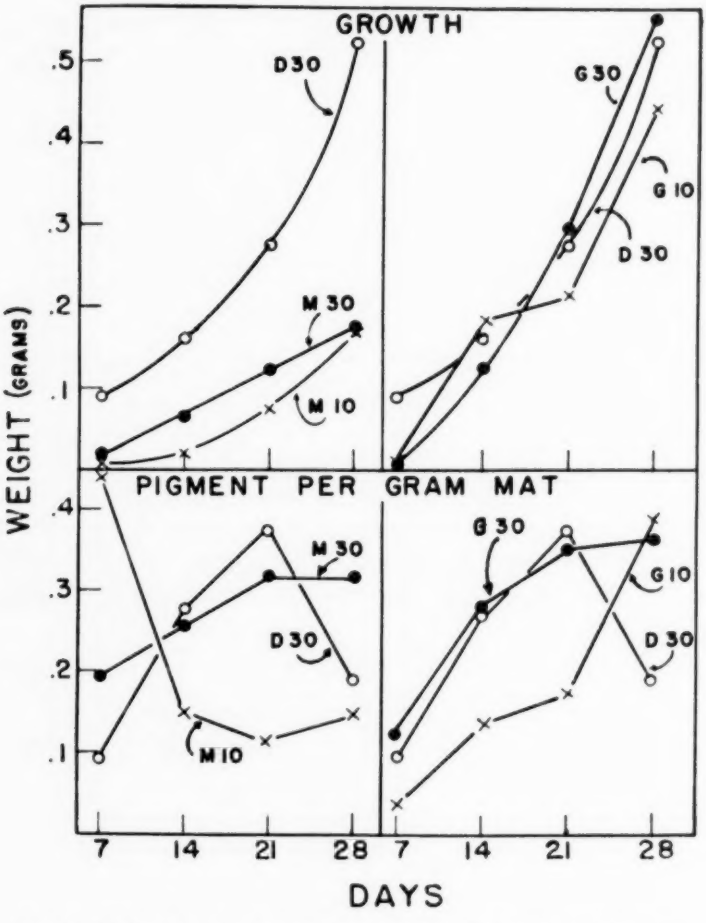

FIG. 4. Effect of carbon sources on growth and pigment production. *A. pseudoglaucus*. Surface cultures.
D = Glucose; M = Mannitol; G = Glycerol.

glycerol and mannitol in pigment synthesis. Despite the fact that growth on mannitol is markedly less, in this experiment production of pigment per gram of mycelium is as good in glycerol and mannitol as in the glucose growths.

Growth and pigment production in submerged culture

In order to avoid the difficulties usually encountered in metabolic experiments involving surface cultures of molds, experiments utilizing submerged cultures were carried out. Some comparisons of typical results which may be obtained with both surface and submerged cultures of *A. novus* and *A. pseudoglaucus* grown on glucose are shown in FIG. 5. In submerged culture the time required for an amount of growth equivalent to that obtained in surface cultures is greatly reduced; growth in submerged cultures at the end of 14 days will appreciably exceed surface growths in most cases. Pigment is produced in submerged cultures but the total pigment produced per culture and particularly the total pigment per gram of growth is markedly reduced in comparison with surface growths. In spite of the decrease in pigment it remains a significant amount. Also, in spite of the poorer pigment production, submerged conditions of cultivation were indicated for further studies because of the desirable characteristics of the growth. In shake flasks, for 3 to 4 days after inoculation, the growth consists of uniform, colorless pellets. Further, beginning about the fourth day, the pellets become colored, starting slowly and increasing in intensity over a four day period. This provides a semi-quantitative index of the rate of pigment production during the course of growth, yielding data which supplement the more quantitative data obtained upon extraction of pigment from the mycelium at the termination of the experiment. Since all pellets are more or less continuously submerged, each pellet on a statistical basis is subject to almost identical conditions of medium, etc. This is to be contrasted with the more variable conditions that obtain with surface cultures where some of the culture is submerged and some is aerial. The submerged growth makes it possible to subject the entire organism uniformly to the selected substrate or inhibitors (to be described below) in a way that is not possible with surface growths.

The results of some further experiments on growth and pigment production in submerged cultures are given in FIG. 6. Here pigment production on glucose and on glycerol are compared under the same environmental conditions. It is striking that, in this experiment, while the pigment per gram of organism is but 10% on glucose, it approaches 60% on glycerol. This suggests again that glyc-

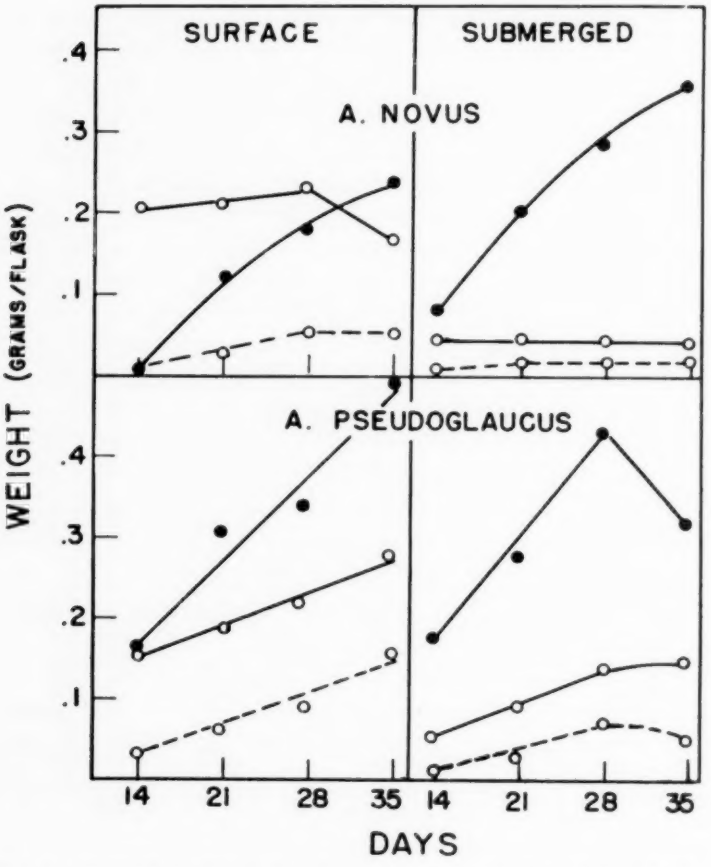

FIG. 5. Comparison of growth and pigment production in surface and submerged cultures. Czapek-Dox medium (10% glucose).
-●——●- = Growth; -○- - -○- = Pigment;
-○——○- = Pigment /gm. mat.

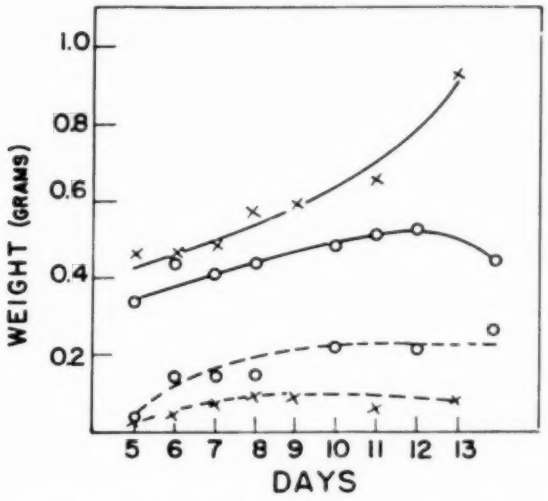

FIG. 6. Growth and pigment production in submerged cultures. *A. pseudoglaucus*. Czapek-Dox medium. × = 10% glucose; ○ = 10% glycerol; ——— = Growth; - - - - = Pigment.

erol or some closely related compound may be particularly significant in pigment synthesis.

Inhibition with fluoride and iodoacetate

The results obtained from a study of pigment production on glycerol suggest that when the organism is grown on glucose the latter may be dissimilated to three carbon residues by the usual glycolytic mechanisms and that these residues may then be built into the more complex pigment molecules. Experiments designed to disrupt the usual pathways for the breakdown of glucose to three carbon residues were set up of which the following is typical. Shake cultures of *A. pseudoglaucus* were allowed to develop for three days until good colorless pellets had been obtained. The inhibitors were added at this time in the concentrations indicated and the cultures were allowed to develop for a total of ten days. At this time growth and pigment production were determined. The results are given in FIG. 7. It is apparent that pigment production occurs on glycerol in the presence of inhibitor concentrations far greater than is

required to inhibit pigment production when glucose is the source of carbon. Both fluoride and iodoacetate stop growth and pigment production on glucose in concentrations that have no effect on the organism developing on glycerol. Experiments which confirm this data and more narrowly delimit the point of pigment inhibition are reported in TABLE II. It can be noted that M/5000 and M/10,000 iodoacetate usually inhibit both growth and pigment production on glucose while M/1000 iodoacetate did not inhibit either growth or

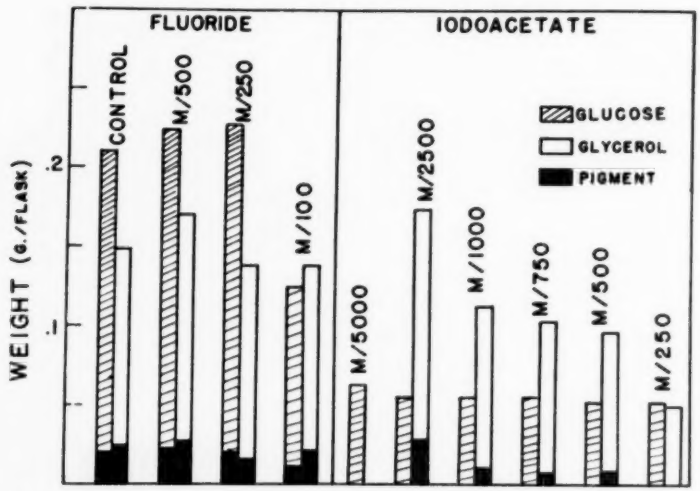

FIG. 7. Effect of sodium fluoride and iodoacetate on growth and pigment production. *A. pseudoglaucus*. Submerged growths. Czapek-Dox medium. Total incubation 10 days.

pigment production on glycerol. Similarly M/250 sodium fluoride inhibited pigment production on glucose while concentrations as high as M/25 fluoride failed to inhibit production on glycerol.

Inhibition of aerobic mechanisms by cyanide and azide and the role of oxygen in pigment production

From the data reported in TABLE II it became apparent that inhibition of the aerobic pathways of carbohydrate metabolism by sodium cyanide or sodium azide has a drastic effect both on glucose

and glycerol metabolism, suggesting that in all probability oxidative metabolism is essential for pigment production. The effects observed with sodium azide were consistently reproducible but those with cyanide were not. Some concentrations of cyanide resulted in good pigment production and there is a suggestion that under some conditions cyanide can be used as a carbon or nitrogen source by the species being studied. Similar results have been obtained in experiments with Fusaria (Gould and Tytell, **1941**).

TABLE II

EFFECT OF INHIBITORS ON GROWTH AND PIGMENT PRODUCTION IN SUBMERGED CULTURES

(*Aspergillus pseudoglaucus*. Data for Growth* on Glucose and Glycerol)

Inhibitor concentration	Sodium fluoride		Iodoacetate		Azide		Cyanide	
	Glucose	Glycerol	Glucose	Glycerol	Glucose	Glycerol	Glucose	Glycerol
M/25	−	+					−	−
M/50	−	+					−	−
M/100	±	+	−	−			−	−
M/250	+	+	−	−			±	−
M/500	+	+	−	−				
M/750			−	±	−	−		
M/1000			−	+	−	−		
M/2500			−	+	−	−		
M/5000			±	+	−	−		
M/10,000			±	+	−	−		
M/25,000			+	+	−	−		

* Medium Czapek-Dox with 10% of carbon source. Ten days' incubation.
\+ Definite pigment production.
− No pigment production.
± Trace of pigment.

To obtain additional data, therefore, the role of oxygen in pigment production was investigated in two additional ways. In the first of these the atmosphere above the organisms growing in submerged culture on the shaking apparatus was replaced by nitrogen gas before pigment production had begun. Both growth and pigment production were effectively stopped (TABLE III). In the second approach two sets of cultures were removed from the shaker before pigment production had begun; a control set was left on. The two sets removed were shaken by hand occasionally to prevent the development of surface growth. Analyses on one of these sets at the end of eleven days (TABLE III) revealed that even though consid-

TABLE III

EFFECT OF ANAEROBIOSIS ON GROWTH AND PIGMENT PRODUCTION
(*Aspergillus pseudoglaucus*. Czapek-Dox Medium)

Conditions of growth	Total growth Dry wt. per flask	Total pigment produced
10% Glycerol		
3 days O_2 + 9 days N_2	.028 g.	None
5 days O_2 + 7 days N_2	.037 g.	Trace only
12 days O_2	.137 g.	10% of growth
10% Glucose		
11 days SG*	.353	8% of growth
3 days SG + 8 days HSG*	.213	Trace only
3 days SG + 8 days HSG + 11 days SG	.352	12% of growth
10% Glycerol		
11 days SG	.250	14% of growth
3 days SG + 8 days HSG	.132	None
3 days SG + 8 days HSG + 11 days SG	.230	12% of growth

* SG = Submerged growth (produced on shaking machine).
HSG = Submerged growth (occasionally hand shaken).

erable growth had been obtained, under such poor oxygen tensions pigment production had been suppressed. This was true in studies with both glucose and glycerol as the carbon sources. At the end of eleven days the second set of hand shaken cultures was returned to conditions of vigorous aeration on the shaker and both growth and pigment production increased markedly during the next eleven days (TABLE III). While these data do not permit a definite conclusion as to whether growth is necessary for pigment production, oxidative metabolism is apparently essential.

Effects of iodoacetate on pigment production on various carbon sources

Experiments were extended to determine the effects of inhibitors on pigment production with carbon sources other than glucose and glycerol. FIG. 8 shows the effect of M/1000 iodoacetate on certain of these. This concentration was selected because at this level pigment production on glycerol is not inhibited. As can be seen, glucose, xylose and fructose cultures were markedly affected; sorbitol and mannitol cultures were affected little or not at all. Also markedly affected (though not indicated in the figure) were the growths on mannose, rhamnose and dulcitol. No growth could be obtained

in either submerged or surface conditions with either pyruvate, lactate, propyl alcohol or ethylene glycol as the sole carbon source in a basal Czapek-Dox solution. Results of further experiments to delimit the concentrations of iodacetate having an inhibitory effect on selected substrates are shown in TABLE IV. Interest in sorbitol and mannitol in connection with pigment production is further enhanced by the fact that the semi-quantitative results indicate that even somewhat higher concentrations of iodacetate are required to inhibit pigment production on these substrates than are necessary for pigment inhibition in glycerol growths.

Experiments with washed resting preparations to study possible pigment precursors

Some slight variability was occasionally noted in the results when attempts were made to determine end points of inhibition such as described. In one experiment, for example, M/750 iodoacetate

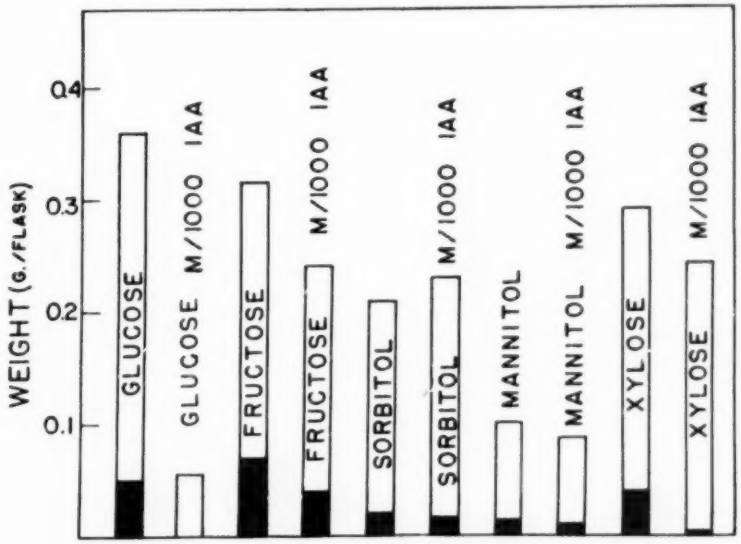

FIG. 8. Effect of iodoacetate (IAA) on growth and pigment production. Various carbon sources (concentrations 10%). *A. pseudoglaucus*. Submerged growths. Czapek-Dox medium. Ten days' incubation. Shaded area indicates pigment. Clear area indicates growth.

would inhibit pigment production on glycerol; in another, a tinge of color might develop and the ether extractions yield minute amounts of pigment. These differences have been ascribed to slight variations in the time of poisoning, small variations that might result in a slightly more vigorous growth, or which might allow the formation of some colorless precursor in the medium prior to the addition of the inhibitor. An experiment was designed to rule out, insofar as possible, the influence of components produced in the medium. For this purpose inoculations were made into media provided with a carbon source known to produce good growth. After three to

TABLE IV

EFFECT OF IODOACETATE ON PIGMENT PRODUCTION
(*Aspergillus pseudoglaucus*. Submerged Growth. Selected Carbon Sources.)
Grown 10 days at 24° C.

Concentration iodoacetate	Glucose	Glycerol	Sorbitol	Mannitol
M/100	−	−	−	−
M/250	−	−	+	−
M/500	−	−	+	±
M/750	−	+	+	+
M/1000	−	+	+	+
M/2000	−	+		
M/2500	−	+		
M/3000	−	+		
M/5000	−			
M/10,000	±			
M/20,000	+			

+ Definite pigment production.
− No definite pigment production.
± Trace of pigment production.

four days, when good colorless pellets had been obtained, the growth was removed, transferred to sterile centrifuge cups provided with sterile screw-top lids, and washed three times with solutions of the same osmotic concentration as those in which they were grown. The resulting mass of growth was diluted just sufficiently to enable it to be taken up, measured and dispersed by means of a sterile large-mouth pipette. Equivalent amounts of washed growth were then transferred to sterile flasks containing the substrates to be tested. When inhibitors were added they were incorporated at this time.

The data presented in TABLE V show the effect of various concentrations of iodoacetate on pigment production of washed glucose and glycerol growths resuspended in various carbon sources as well as in growth media, as indicated. The previous trends in the data are again confirmed. Pigment production on glucose is inhibited by concentrations far too weak to affect production on glycerol. Glycerol, sorbitol and mannitol behave as they did before. A striking observation is that the original growth medium was capable of supporting pigment production at unusually high inhibitor concentrations, either when the original medium contained glycerol or glucose.

TABLE V

EFFECT OF IODOACETATE ON PIGMENT PRODUCTION OF WASHED GLUCOSE AND GLYCEROL GROWTHS RESUSPENDED IN VARIOUS CARBON SOURCES AS INDICATED

(*Aspergillus pseudoglaucus*. Submerged Cultures)

Iodoacetate	Glucose growths					Glycerol growths				
	Old Medium*	Glucose	Glycerol	Sorbitol	Mannitol	Old Medium*	Glucose	Glycerol	Sorbitol	Mannitol
M/1000	−	−	−	−	−	+	−	−	−	−
M/2000	−	−	−	−	−	+	−	+	−	−
M/3000	±	−	±	±	−	+	−	+	−	−
M/5000	+	−	+	+	+	+	−	+	+	+
M/10,000	+	−	+	+	+		−			
M/20,000	+	+	+	+	+		+			

* Old Medium = the fluid in which the washed growth had grown.
+ Pigment produced; − No pigment produced; ± Trace of pigment production.

This is highly suggestive of the possibility that the growth medium contains probable precursors of the pigments. An examination of the data indicates that glucose growths replaced on old medium containing M/5000 iodoacetate produced pigment, while they did not when placed on fresh glucose medium containing M/10,000 iodoacetate. On the other hand glycerol growths placed on old glycerol medium could produce pigment at M/1000 iodoacetate as compared with no pigment production at M/10,000 iodoacetate when glycerol growths were placed on fresh glucose medium. If the organisms contained all essential precursors for the pigments this would not be the case. It appears therefore that the pigment

TABLE VI

INFLUENCE OF THE GROWTH MEDIUM IN RELATION TO THE EFFECT OF M/2000 IODOACETATE ON GROWTH AND PIGMENT PRODUCTION
(*Aspergillus pseudoglaucus*. Submerged Cultures. Washed Glycerol Growths)

New suspending medium	Pigment fifth day of growth	Pigment sixth day of growth	Pigment seventh day of growth	Total growth Dry weight grams (13 days)	Pigment per gram of growth (Dry wt. basis) (13 days)
Unwashed Control	−	+⁺	++⁺	0.370	.100
75 ml. OM (Old Medium)*	−	±	++	0.763	.080
50 ml. OM + 25 ml. Glycerol	−	±	++	0.624	.042
25 ml. OM + 50 ml. Glycerol	−	±	+⁺	0.676	.086
15 ml. OM + 60 ml. Glycerol	−	−	+⁺	0.563	.048
5 ml. OM + 70 ml. Glycerol	−	−	±	0.482	.046
0 ml. OM + 75 ml. Glycerol	−	−	−	0.178	.000

* OM = Old Medium = the fluid in which the washed growths had grown.
− no pigment. + definite pigment. +++ most strongly pigmented.
± trace of pigment. ++ strongly pigmented.

precursor is associated with some soluble metabolic intermediate in the medium and is not concerned solely with components of the growth itself.

Additional evidence that substances in the original growth medium stimulate pigment production was obtained from experiments

TABLE VII

EFFECT OF VARIOUS CARBON SOURCES ON PIGMENT PRODUCTION THREE DAY WASHED GLUCOSE GROWTHS RESUSPENDED IN SUBSTRATE INDICATED
(*Aspergillus pseudoglaucus*. Submerged Cultures)

Substrate	Pigment on fourth day	Pigment on fifth day	Pigment on sixth day
5% Glycerol	−	+⁺	++
5% Sorbitol	−	+⁺	+⁺
5% Mannitol	−	+⁻	+⁻
5% Fructose	−	−	−
5% Glucose	−	−	−
5% Glucose + 5% Glycerol	−	+⁻	++
5% Glucose + 5% Sorbitol	−	+	++
5% Glucose + 5% Mannitol	−	+	+
5% Glucose + 5% Fructose	−	+⁻	+⁻
10% Sucrose	−	−	−
10% Glucose	−	±	+

− No pigment.
± Trace of pigment.
+ Definite pigment; +⁻ slightly less intense.
+⁺ Strongly pigmented but not as strong as ++.

of the type whose results are recorded in TABLE VI. Here glycerol growths were washed by centrifugation, as before, and resuspended in 75 ml. of glycerol medium. Pigment production on this medium was compared with that on various combinations of the new glycerol medium and the old glycerol medium in which the washed growths had been produced. The use of glycerol as a substrate results in considerable pigment; therefore these growths were partially poisoned with M/2000 iodoacetate to extend the time required for such pigment production as did occur. Both the qualitative and quantitative data show that growth and pigment production are directly related to the amount of old glycerol medium that is provided, suggesting that the old growth medium contains some precursor of the pigment or a factor that aids in its production.

TABLE VIII

PIGMENT PRODUCTION BY WASHED SUBMERGED GROWTH ON VARIOUS SUBSTRATES

Aspergillus pseudoglaucus Pigment per gram of growth (dry weight)

	Glycerol growths[1]	Glycerol growths[2]	Glucose growths[2]	Glucose growths[2]	Average of four experiments
Old Medium[3]	0.290	0.146	0.122	0.172	0.183
Glycerol	0.198	0.122	0.239	0.200	0.190
Mannitol	0.178	0.078	0.076	0.173	0.126
Sorbitol	0.184	0.047	0.084	0.134	0.112
Glucose	0.108	0.066	0.103	0.149	0.107

[1] Seven days on new substrate.
[2] Four days on new substrate.
[3] Old Medium—the fluid in which the washed growths had grown.

Attempts to determine whether the factor might be one of the compounds previously studied were made by resuspending the washed growths on the various substrates suggested by the data. The results of such an experiment are recorded in TABLE VII. Glycerol, sorbitol and mannitol were found to be more effective in stimulating pigment production than other carbon sources tried. Such experiments have been placed on a quantitative basis and the results recorded in TABLE VIII. Here data have been obtained for two typical experiments using glycerol growths; two experiments using glucose growths, and the averages are given for the four attempts in which such an experiment was tried. Again glycerol,

mannitol, and sorbitol, in the order cited, seem the best pigment-producing substances found.

DISCUSSION

The experimental results above reported support certain suggestions as to the probable pathway by which strains of *Aspergillus* synthesize complex pigment molecules such as auroglaucin and flavoglaucin from glucose. The experiments with inhibitors suggest that reactions common to carbohydrate breakdown in yeast and animal tissue are also involved in pigment synthesis by members of the *A. glaucus* group. The results supporting this hypothesis are particularly striking since the concentration of inhibitor found effective in preventing pigment formation on glucose is of the same order as the concentration found effective in inhibiting glycolytic reactions with yeast and animal tissue preparations.

Further, pigment production from glycerol is not inhibited by concentrations of these inhibitors considerably higher than those which affect glucose metabolism. This suggests that once the sugar has reached the three carbon stage certain inhibitors of carbohydrate breakdown do not block pigment synthesis. In this connection it may be noted that pigment production from xylose, which is probably converted to a two and three carbon residue by a different route from that which glucose follows, is less affected by the inhibitors than is glucose.

The results strongly suggest that mannitol or sorbitol may be an intermediate in the synthesis of the pigments. This is particularly interesting since Yamasaki and Simomura (**1937**) have previously proposed that glycerol is a direct precursor of mannitol on the basis of experiments in which they were able to obtain 20–30% yields of mannitol from glycerol as a substrate using strains of *Aspergillus glaucus*. In our experiments mannitol and sorbitol often stimulated pigment production even more than glycerol, also suggesting the possibility of resynthesis of three carbon residues to six carbon residues. Furthermore, the fact that pigment production on mannitol could usually be carried on at higher concentrations of iodoacetate than those which inhibited pigment production on glycerol suggests that the three carbon residues are built into the larger residues

rather than the mannitol being degraded to the three carbon compound which is then utilized in pigment synthesis.

The data presented suggest the following tentative pathway for pigment synthesis. Glucose is converted by the usual pathway of carbohydrate dissimilation to the three carbon stage; the three carbon compound is then perhaps condensed to a six carbon residue such as mannitol which then is converted in some manner to the pigments. No conclusive evidence is available as to whether glycerol itself is the three carbon compound that is involved, but it is interesting that neither pyruvate nor lactate enhances pigment production.

The fact that oxygen is necessary for pigment production from both glucose and glycerol suggests that, assuming glycerol to be an intermediate in pigment production, oxygen is involved in the subsequent synthesis of the three carbon residues to the more complex molecule.

The possible role of mannitol as an intermediate in the formation of mold metabolic products is extremely attractive in view of the ubiquitous nature of the compound in mold metabolism as well as the fact that some of these organisms are known to produce extremely large quantities of it.

SUMMARY

(1) Auroglaucin and flavoglaucin are major end products of carbohydrate metabolism among members of the *Aspergillus glaucus* group. Striking quantities of these pigments are produced in both surface and submerged cultures.

(2) Even after considerable growth has developed, aerobic conditions are essential for pigment formation.

(3) In surface culture, organisms grown on glycerol, mannitol and xylose produced almost twice the amount of pigment per gram of mycelium as those grown on glucose. In submerged culture, organisms grown on glycerol produced even higher ratios of pigment per gram of mycelium than comparable glucose growths.

(4) In a basal Czapek-Dox solution no growth could be obtained in surface or submerged culture with either pyruvate, lactate, propyl alcohol or ethylene glycol as the sole carbon source.

(5) In submerged culture M/5000 and M/10,000 iodoacetate added the third day of growth inhibited both growth and pigment production on glucose while M/1000 iodoacetate inhibited neither growth nor pigment production on glycerol.

(6) In submerged culture M/250 sodium fluoride inhibited pigment production on glucose while concentrations as high as M/25 failed to inhibit production on glycerol.

(7) M/1000 iodoacetate markedly affected growth and pigment production on glucose, mannose, fructose, xylose, rhamnose and dulcitol; growth and pigment production on glycerol, mannitol and sorbitol was affected little or not at all.

(8) Pigment production by washed "resting preparations" on glucose is inhibited by concentrations of iodoacetate far too weak to inhibit production on glycerol, sorbitol and mannitol.

(9) Soluble intermediates in the growth medium may play a significant role in pigment production.

(10) The data presented suggest that the following tentative pathway may be involved in pigment synthesis by members of the *A. glaucus* group. Glucose is dissimilated by the usual route of carbohydrate dissimilation in yeast to the three carbon stage; the three carbon compound is then condensed to a six carbon residue such as mannitol, which is then converted in some yet undetermined manner to the pigments.

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ZONATION IN A PROLINELESS STRAIN OF NEUROSPORA [1]

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(WITH 3 FIGURES)

There has been a considerable amount of investigation concerning the phenomenon of zoning, *i.e.*, the formation of regular differences in types of growth of fungi in culture, giving the appearance of concentric rings in petri dish cultures and regular bands in horizontal tubes. Most workers have attributed this to variations in the environment: Bisby (**1925**), Brown (**1925**), Gallemaerts (**1912**), Hedgecock (**1906**), Hall (**1933**), Hutchinson (**1906**), Knischewsky (**1909**), Molz (**1906**), Rahn (**1912**), and Snyder and Hansen (**1941**) found that alternation of light and dark caused zonation in their various test organisms. Temperature variation was shown to be the factor causing zonation in organisms tested by Bisby (**1925**), Ellis (**1931**), and Hall (**1933**). Munk (**1912**) found that fluctuations in humidity caused zonation of the fungi with which he was working. There has been some evidence, however, of the existence of another type of zonation, namely that which takes place in the absence of environmental fluctuations or which at least cannot be correlated with them. Hall (**1933**) states that: "Earlier workers have clearly recognized two types of successive zoning—first, a type which arises under uniform conditions of environment and, second, a type in which the zoning is correlated with a periodicity of the environment." Hedgecock (**1906**) mentions the first type and states: "This type has been observed by the writer in a number of species of fungi; among these is *Hypocrea*. Thomas Milburn mentions this type of ring formation in his studies of *Hypocrea rufa* and other fungi. In *Hypocrea* the concentric rings are formed, not daily, but in a much longer period

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of time." More recently, Brown (**1925**) noted something perhaps similar and said that some strains of *Fusarium* form one strong zone of spores which obviously has no relation to the periodicity of light. Stevens and Hall (**1909**) found that zonation of their test organism occurred either in constant light or constant dark and thought that this might be due to crowding of the mycelium.

The writer first noticed zonation in *Neurospora crassa* strain *21863*, while using it as a test organism in routine laboratory experiments. To his knowledge the phenomenon has not been previously reported in any strain of the species. At the time it was first observed, the tube technique of Ryan, Beadle and Tatum (**1943**) was being used and the zoning was manifested as alternated bands of dense and sparse formations of conidia. The writer thought it might be interesting to try to find the cause of zonation in this particular case; hence the following study was undertaken.

MATERIALS AND METHODS

Since the phenomenon was originally observed in tubes, and the organism grows at too rapid a rate to make petri dish culture practical, tubes were used throughout this investigation. In addition the tubes are generally easier to work with. The medium, also used throughout, was one devised by Gray (**1946**) and contained the following: 5 g. dextrose, 0.7 g. Difco yeast extract, 0.5 g. KH_2PO_4, and 2 g. agar per 100 ml. water. The liquid medium was pipetted into tubes which were plugged and placed in an autoclave. Changes take place in this medium during autoclaving, so it is desirable to keep the length of time in the autoclave about the same for each batch of medium in order that comparisons between experiments can be made. In this work a thirty minute autoclaving at fifteen pounds pressure (121° C.) was used; exceptions are so noted.

The portions of this research carried out under controlled conditions were done in a subterranean laboratory which was windowless, well insulated, and had a tight-fitting, refrigerator-type door. It was possible to maintain the desired temperatures within a range of $\pm \frac{1}{2}$° F. and relative humidity within a range of ± 2 per cent. A hygrothermograph recorded temperatures and relative humidities.

The source of light for this investigation was a single 60-watt

Westinghouse "daylight" bulb (blue coating) in a white metal shade, situated to furnish an intensity of illumination of about 15 to 25 foot candles. A dark box consisting of a box within a box was constructed of corrugated cardboard and placed immediately adjacent to the illuminated area. It had an opening four inches by four inches to allow air circulation so that the temperature in the box would not be too different from that outside. A maximum-minimum thermometer was kept inside the box during each experiment; it was found that the temperature inside the box was always within 1° F. of the temperature outside. The box was tested for possible light leaks by exposing photographic film (Eastman type B, press—Weston rating undetermined) inside the box for ten minutes while the light was turned on outside. When the film was developed, it showed no fogging, indicating there were no light leaks of sufficient intensity to cause a reaction of the emulsion.

The most accurate and practical method of recording results was deemed to be photographs of the culture-containing tubes taken at the end of each experiment. These were taken with light transmitted through the tubes as this method was found to give photographs of greater detail than if reflected light were used.

RESULTS

Investigation of Possible Zonation in Various *N. crassa* Strains

As zoning was observed in strain *21863*, it was thought that the phenomenon might occur in other strains of *N. crassa*. The following strains were therefore cultured in the laboratory under the same conditions in which the author first observed zonation in strain *21863*, namely, the diurnal variations in light, temperature and humidity to which the laboratory was ordinarily subjected. Note that some pigmented and some non-pigmented strains were used.

Strain	*Description*
21863	—pigmented, prolineless (unable to synthesize proline); this mutant is the test organism used in other portions of the study.
33757, 4637	—non-pigmented, leucineless.
15300	—an albino mutant.
34105	—pigmented, able to utilize ornithine, citrulline or arginine.
27947	—pigmented, able to utilize ornithine, citrulline or arginine.
4545	—pigmented, lysineless.

The light impinging upon cultures of these strains was of varying and unknown intensity and consisted of indirect sunlight and/or light from overhead lamps. A hygrothermograph recorded the temperature which varied from 79° F. to 84° F., and relative humidity which varied between 22 per cent and 38 per cent.

Several days later it was obvious that zonation had occurred only in the tubes containing strain *21863*.

Later, other strains of the ornithine cycle (Srb *et al.*, **1944**) were tested but none exhibited zoning. This does not imply that zoning would not occur under the proper conditions; a combination of inheritance factors similar to that responsible for zoning in strain *21863* might be present in one or more of these strains, its expression being masked by unfavorable (relative to zoning) environmental circumstances. The other strains tested were:

36703—argininеless
30300—citrulline-argininеless
33442—citrulline-argininеless
29997—ornithine-citrulline-argininеless

The remainder of the strains of this cycle as propounded by Srb, Fincham and Bonner (**1950**) were not tested since they were not immediately available.

Zonation Under Controlled Conditions

Since the previously mentioned zones appeared to correspond approximately to diurnal variations in light, an experiment was performed to study the effect of light on conidia formation. Fourteen tubes containing Gray's medium were inoculated with the test organism and placed in the controlled conditions room mentioned above. During this run, the temperature was 89° F. $\pm$ ½° F. and the relative humidity was 64 per cent $\pm$ 2 per cent. The lamp mentioned above was turned on so that germination and initial growth took place in the light. When the mycelial front had advanced a few millimeters, the position of the front was marked on each tube with a wax pencil and three tubes were placed in the dark box. Six hours later, the location of the mycelial front was again marked on the tubes remaining in the light and three more tubes were placed in the dark box. This was repeated with a

third and fourth set of three tubes each. Two tubes were left in the light to serve as controls. Ninety-six hours later, all tubes were removed from the dark box and the positions of mycelial fronts marked (FIG. 1). Zonation had taken place in all tubes which had been under the dark box and alternate bands were approximately equidistant in all tubes. It is evident from FIG. 1 that light inhibited zonation and that zonation occurred in each tube following the elapse of approximately the same time interval after being transferred to the dark. Once zonation occurred, however, the positions of the alternate bands appeared to be independent of the environment.

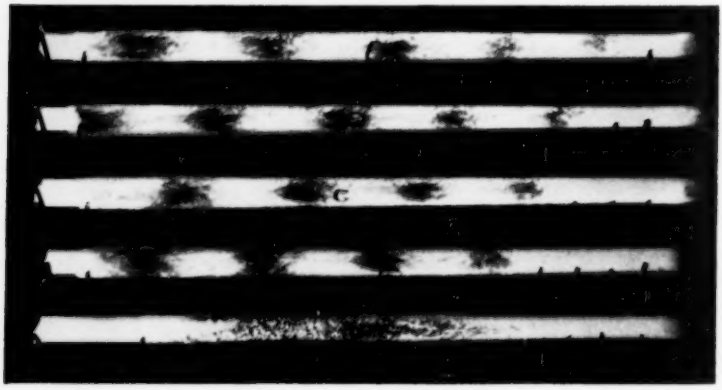

FIG. 1. Zonation under controlled conditions. Tube C was the first placed in the dark; tubes D, C and K were then placed in the dark 6, 12 and 18 hours respectively after C. Tube N was under continuous light. Note zoning in all tubes but N, and retarding of growth in tube N.

In these tubes a line connecting centers of zones was diagonal in relation to the tubes of different sets. Had fluctuations in any environmental factor been responsible for the zoning, a line connecting centers of zones would have been perpendicular to the tubes if they were placed parallel with all the first wax marks lined up.

Although in this experiment the conidia germinated in the light, and the tubes were subsequently placed in the dark, preliminary experiments had demonstrated that zonation will occur if germination of the conidia and subsequent growth of the mycelia occurs in total darkness.

Relation of Intensity of Illumination to Linear Growth

Beadle and Tatum (**1945**) stated that light intensity had no effect on the rate of linear growth of their test organisms, which were also strains of *N. crassa*. The author has noticed, however, that in addition to inhibiting zonation, continuous light seemed to slow down linear growth of strain *2186.3*. This experiment was performed to study the effect of light intensity on linear growth of the mycelium. Gray's medium was poured into three tubes, each about one meter long. Two of the tubes were placed under the same light source and moved during the course of the run so that the foremost 100 mm. of the mycelium was exposed to an intensity of illumination of 22 to 24 foot candles. The remaining tube was so placed that it received an intensity of illumination ranging from 0.04 foot candle at one end of the tube to 0.07 foot candle at the other end. Intensity of illumination was measured with a MacBeth Illuminometer, using the white disc placed at the same level as the tubes. The temperature range during the course of this run was 89° F. $\pm \frac{1}{2}$° F. and the relative humidity range was 66 per cent $\pm$ 2 per cent. The position of each mycelial front was marked daily and charted. While the light remained on, no zoning occurred in any of the tubes. A little more than sixteen days after the tubes were inoculated the light burned out and the tubes were left in the dark for three days, during which time no zoning took place in the tube which had been under the lower illumination, although slight zoning was apparent in the tubes which had been under higher illumination. In sixteen days in the light, the mycelium advanced 780 millimeters from the first mark in both tubes which had been exposed to the higher illumination while it advanced 887 millimeters in the tube exposed to the lower illumination.

Length of Dark Period Necessary to Permit Zonation

Since zoning occurred in continuous darkness, was inhibited by continuous light, but in preliminary experiments occurred under alternative light and dark periods each of 12 hours duration, it was inferred that there must be a minimum dark period which will permit zoning. It was further inferred that this minimum dark period must be within the range of 0–12 hours per 24-hour cycle.

Nine tubes, containing Gray's medium were inoculated with strain *21863* and placed in the dark in the controlled conditions room. During this run the temperature was 88.5° F. ± ½° F. and the relative humidity was 66.5 per cent ± 1.5 per cent. When the mycelial front had advanced a few millimeters, its position was marked on each tube. Three tubes were then placed under the dark box for a 2-hour period, three more for a 4-hour period and

FIG. 2. Length of dark period necessary to permit zonation. Tubes D, E, F were subjected to 2 hours of dark per day. Tubes G, H, I, to 4 hours of dark per day. Tubes A, B, C, to 6 hours of dark per day. When not in the dark, these tubes were subjected to light which had an intensity of illumination of 15 to 25 foot candles.

three more for a 6-hour period and then returned to the light. This procedure was repeated daily, one set of tubes thereby receiving 2 hours of darkness plus 22 hours of light, a second set 4 hours of darkness plus 20 hours of light, and a third 6 hours of darkness plus 18 hours of light. When the mycelial front had nearly reached the opposite end of the tubes, the results were recorded (FIG. 2). There was obvious zonation only in the tubes

receiving six hours of darkness. No obvious zonation occurred in either the set receiving two hours of darkness or the set receiving four hours of darkness, although there was perhaps a slight hint of zoning in the latter set, indicated more by slight difference in color and structure of the mycelium than by slight differences in its density. On the basis of these data the minimum dark period for obvious zonation under these conditions is of four to six hours duration.

Relations of Media to Zonation

A preliminary run made in the earlier stages of the investigation had shown that a sugar concentration of 5 per cent was better as far as zoning was concerned than 0.5 per cent or 10 per cent, so 5 per cent was the concentration used throughout the work. The effect of various kinds of media on zonation was also investigated. These media were: prune agar, nutrient agar, lima bean agar, corn meal agar, brain-veal agar, beef-lactose agar (all Difco products), potato-dextrose agar (200 g. potato tuber, 20 g. dextrose, 17 g. agar, 1 liter of water), and two lots of Gray's medium, one autoclaved for 30 minutes and the second autoclaved for 90 minutes. The clue that autoclaving this medium more than 30 minutes had some effect upon its composition was provided when tubes were inadvertently left longer than this period in the autoclave. When the tubes were finally removed, it was observed that a decided color change in the medium had occurred. Upon cooling, the agar did not form as firm a gel as that which was autoclaved for only 30 minutes. This medium was included in the experiment to ascertain whether or not any changes had occurred in the medium which would significantly affect zonation. Three tubes of each medium were inoculated with strain *2186.3* and placed in the controlled conditions room with the light on.

When the mycelial front had advanced a few millimeters, its position was marked on the tubes and the light was turned out. Five days later the position of the mycelial front was again marked and the results were recorded (FIG. 3). Obvious zonation occurred only in the tubes containing Gray's medium—the zones being a little better defined on the medium which had been auto-

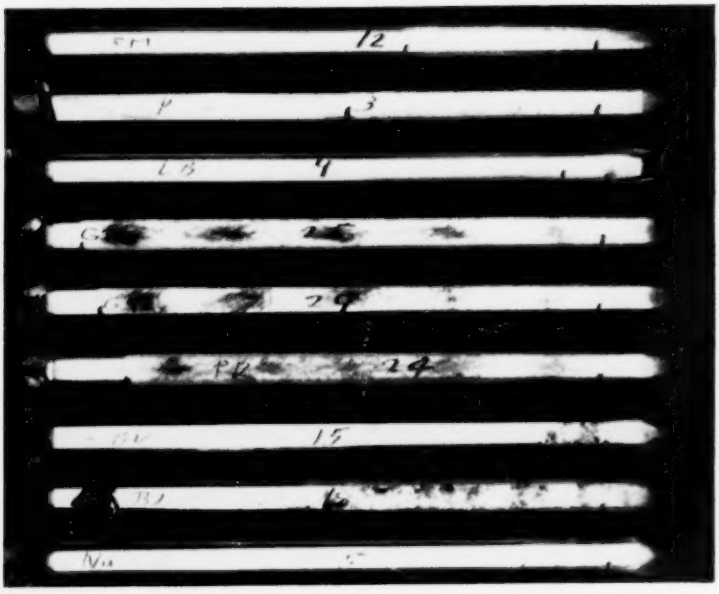

FIG. 3. Relations of media to zoning. On the above tubes, the labels denote the following: CM—corn meal agar; P—prune agar; LB—lima bean agar; G3—Gray's medium autoclaved 30 minutes; G9—Gray's medium autoclaved 90 minutes; PD—potato-dextrose agar; BV—brain veal agar; BL—beef lactose agar; Nu—nutrient agar. The wax pencil marks on the left denote the position of the mycelial front.

claved 90 minutes. Zonation on other media was poor or not present at all.

The Relation of Proline to Growth of Strain *21863*

In order to be sure that strain *21863* was still prolineless and had not lost this character in culture, and to see what effect, if any, different concentrations of proline might have on zonation and linear growth, a series of tubes was set up containing a minimal medium plus varying concentrations of proline. This medium is similar to that described by Beadle and Tatum (**1945**) in their work on nutritional requirements.

The writer arbitrarily selected the following amounts of proline: 0 mg., 1 mg., 2 mg., and 4 mg. per 100 cc. medium. Three tubes

were half-filled with each of these media and inoculated with strain *21863* and placed in a dark room containing no temperature or humidity control equipment. However, a hygrothermograph was placed immediately adjacent to the tubes and recorded a temperature range of 83–87° F. and a relative humidity range of 24–32 per cent.

Nine days later, the tubes were observed, and the results recorded. In the tubes containing no added proline, germination and growth was limited to small tufts, none of which exceeded 5 mm. diameter. In the other tubes, containing various concentrations of added proline, the mycelium had grown about 250 mm. in most cases. The rate of linear growth seemed to be about the same regardless of the concentration of proline but the amount of mycelium appeared to increase directly with proline concentration.

There was no zoning in any of these tubes, the growth appearing irregular. There were, however, in the tubes containing 4 mg. proline per 100 cc. medium, scattered patches which had the general appearance of the type of growth in the dense area of a zone. In general the growth was different from the type of growth obtained under continuous light. It appears from these observations that there was some ingredient which is required for zoning lacking from the medium or perhaps it was not present in the proper quantity. It is also possible that some constituent of the medium inhibited zonation.

DISCUSSION

Zonation in strain *21863* seems to be a different sort of phenomenon than that described by most previous investigators, although it is possible that it is similar to that described by Hedgecock (**1906**), Millburn (**1904**) and Brown (**1925**). It is doubtful that it is related to the type discussed by Stevens and Hall (**1909**), since zoning in their test organism seemed to be due to alternate concentric areas of dense and sparse mycelium, whereas in strain *21863* the zoned appearance was due to alternate bands of dense and sparse conidia. The writer was unable to find an instance where either Hedgecock, Millburn or Brown carried out controlled experiments which definitely showed that their particular test organism zoned under relatively constant conditions. This would

have been reasonably easy to show had the tube technique been known. This technique offers excellent possibilities of finding the exact chemical and physical processes which result in zoning in strain *21863*.

Most of the experiments which were completed in this investigation are primarily exploratory and the openings for additional experimentation are abundant. One striking possibility for further investigation is that perhaps this phenomenon is related to the ornithine cycle, or if not, it may prove to be a useful character in the genetic phase of this investigation.

Although the writer feels premature in proposing a possible mechanism for zoning in strain *21863*, the following hypothesis is offered in an attempt to relate data available at this stage of investigation.

Consider a tube recently inoculated with strain *21863* which has been under light until the colony reaches whatever size and physiological state is necessary for zoning to occur. When the light is turned out, a certain series of events take place, namely: the colony continues growing down the tube for about six hours (89° F.) with no noticeable increase in its density; then a very apparent dense area of conidia and aerial hyphae is formed for several hours; this type of growth then ceases and the former sparse type resumes for several hours until the dense type starts again, etc. Suppose that when the light is turned out, a chain of reactions is able to go to completion which results in zoning; since zoning doesn't occur when the light is left on, at least one substance produced in this chain must be light labile, and the chain is thus blocked by the light.

Postulate further the production and accumulation in the course of chain "α" of a light stable substance D which is necessary for the fabrication of the dense zone. That this substance is light stable is demonstrated by the observed fact that light applied just after the dense zone begins to form doesn't inhibit the formation of the rest of the zone. The zone will continue to form in the light as long as 6 to 10 hours after the application of light.

However, even if no light is applied, the formation of the dense region eventually ceases. To explain this phenomenon a substance "X" is hypothesized which is produced by a side reaction during

the preceding series of events which inhibits conidia formation when present in sufficient concentration—further, it inhibits the resumption of the chain of reactions "α" until the mycelium grows beyond its influence. Then, if no light is present, chain "α" starts again and in about six more hours, the dense zone is started once more.

Assume that the size of the dense area is proportional to the amount of D synthesized; then if light is applied soon after a small amount of D is produced, the dense area should not be very dense and not very obvious. This is the case where tubes were subjected to only four hours of darkness—when they got six hours, the zones were quite obvious. No zones could be detected where the tubes received 2 hours of darkness and it is inferred that the reactions resulting in D had not had time to go to completion before light destroyed the labile precursor, while in the tubes receiving 4 hours of darkness, the reaction apparently had produced a small amount of the stable D before the light inhibited its further production. From these observations, it is inferred that under the conditions of this investigation, the reactions producing "D" were between 2 and 4 hours long, seemingly closer to 4 hours than two.

Since the first conidia of the dense zone seemed to appear about six hours after the inhibitory effect of either light or factor "X" were removed, it is inferred that the reactions starting with "D" as a substrate go to completion with the formation of structures of the dense zone in about 2 (perhaps somewhat more) hours. That is, 6 hours minus the time required to produce D.

If the light is not turned off at the time indicated at the beginning of this discussion, no zonation will take place; that is, there is no alternate formation of dense and sparse zones. However, under continuous illumination, conidia and aerial hyphae do begin to form in abundance approximately 18 (± 6) hours after the colony is physiologically capable of forming a dense zone in the dark. The chain of reactions resulting in non-zonation in continuous light will be referred to as chain β. Since a different structure results in continuous light, it is inferred that a different process must be involved, at least in part. It is apparent in FIG. 2 that there is a considerable amount of rather dense growth between the so-called "dense areas." It appears that chain β is taking place in the

tubes which received 2 hours of dark and that perhaps it is still taking place to some extent in the other tubes and that the only difference in chains of reaction α and β is the production of "X." The writer would be inclined toward this point of view were it not for the observation that the appearance of dense growth produced by α is much different from that of chain β. Which is the valid point of view could perhaps be decided by microscopic examination of hyphae and spore structure but this was impossible with the type of tube used.

If the above hypothesis is correct, manipulation of the zones should be possible. Preliminary tests indicate this to be the case. Application of light to the colony before the inhibitory effect of "X" had dissipated prevented dense areas from forming after the usual interval of sparse growth. Regular zoning was resumed when the light was removed after a 48-hour period of sparse growth. In another preliminary test, the length of dense and sparse zones was cut down by light manipulation so that five dense zones were formed in the test colonies in the same time that four dense zones were formed in the controls. However, neither of these tests was performed in a critical manner, and the results should be interpreted as indicatory only.

SUMMARY

1. *Neurospora crassa* strain *21863* zoned under certain conditions.

2. Nine other strains of the same species did not zone under the conditions which permitted zoning in strain *21863*.

3. Under constant environmental conditions, strain *21863* zoned in continuous darkness.

4. Under constant environmental conditions, strain *21863* did not zone in continuous light.

5. The minimum dark period per 24-hour cycle which permitted obvious zoning was of 4 to 6 hours duration.

6. The medium on which zoning was most distinct (of the media tested by the writer) was Gray's medium autoclaved for 90 minutes, and was almost as distinct on the same type of medium autoclaved for 30 minutes.

7. Continuous light resulted in a reduction in rate of linear growth; 22–24 foot candles retarded linear growth more than 0.04–0.07 foot candles.

8. Intensity of illumination as low as 0.04–0.07 foot candles inhibited zonation when supplied continuously at 89° F. and relative humidity 66 per cent.

The writer wishes to express his sincere appreciation to Dr. W. D. Gray for his suggestions, technical assistance, and for crictically reading this manuscript.

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PHAGE PROBLEMS IN THE STREPTOMYCIN FERMENTATION

FERNANDO CARVAJAL [1]

(WITH 3 FIGURES)

Since 1915 and 1917 when Twort (**12**) and d'Herelle (**5**) discovered the phenomenon of bacteriophagy, interest has been focused on the nature of the phage, its reproduction and the mechanism of its action upon the host cell. The phages of bacteria and Actinomycetes have most of the characteristics usually attributed to the animal and plant viruses.

Since the discovery of the phage of *Streptomyces griseus* in 1947 by Saudek and Colingsworth (**10**), similar outbreaks of the phage were reported from other sections of this country and later on from other countries. These outbreaks have resulted in considerable loss of streptomycin production to the industry. The phage of *Streptomyces griseus* has been called an *Actinophage*, though it should more properly be named a *Mycophage* due to the nature of *Streptomyces griseus*, which is a typical fungus (**2, 3**). For simplicity in the present publication it will be referred to as phage of *Streptomyces griseus*. The term *Mycophagy* indicates the phenomenon of host cell disintegration by the action of the phage.

Since 1945 we have been studying fermentations with *Streptomyces griseus* for the production of streptomycin. However, it was not until March, 1952, that we encountered any phage outbreak. This led us to a more detailed study of the whole phage problem in streptomycin fermentation. The sources of the phage used in all of the work reported in this communication were infected tanks at our commercial plant. The *Streptomyces griseus* used was the

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culture *U-203,* a mutant produced by ultraviolet light irradiation of the original culture *SL-842* which was isolated from Ohio River mud in our laboratories in March, 1945 (**4**).

PHAGE IN THE ACTINOMYCETES WITH SPECIAL REFERENCE TO THE MAIN PUBLICATIONS ON *STREPTOMYCES GRISEUS* PHAGE

In 1936 Wiebols and Wieringa (**17**) were the first clearly to prove the existence of phage in the group Actinomycetes. They called this phenomenon *Microbiophagy.* They found phages in species of *Streptomyces,* including *S. scabies* obtained from infected potatoes. They also found phages from the pathogenic *Actinomyces bovis* and *Nocardia farcinica.* They produced the typical plaques and lysis due to the phage action upon the various species of Actinomycetes comparable to those caused by bacteriophages upon bacteria. They also reported a phage with the ability of lysing more than one species of Actinomycetes, *i.e.,* a polyvalent phage.

Saudek and Colingsworth (**10**) were the first to report, in 1947, the discovery of a phage for streptomycin-producing strains of *Streptomyces griseus* in their fermentations. The phage propagated rapidly in young cultures of *Streptomyces griseus,* bringing about the lysis of the organism. Cultures resistant to the phage were isolated for streptomycin production. When the phage was added to a fermentation with the sensitive strain of *Streptomyces griseus,* production of streptomycin was partially or completely prevented. The plaque method was used to measure the amount of phage particles present in the broth.

In 1947 Woodruff *et al.* (**18**) isolated a phage in laboratory stationary liquid cultures of *Streptomyces griseus* in flasks which were exposed to laboratory air for 24 hours. They reported that the strains of *Streptomyces griseus* which do not produce streptomycin were resistant to the action of the phage and that only the streptomycin-producing strains were sensitive to the phage. They also reported that six additional species of *Streptomyces* were not affected by the phage. Studying the morphology of the phage in the electron microscope, they found the phage to resemble *Escherichia coli* phage. They described it as follows: the majority of the heads were symmetrically spherical, 0.05 μ in diameter; tail

bent, long, relatively thick, about $0.15 \times 0.015\,\mu$. Many phage particles consisted of two bodies and a few were tetrads.

In 1947, Reilly, Harris and Waksman (**9**) fully confirmed the previous findings of Saudek and Colingsworth (**10**) and Woodruff *et al.* (**18**). Reilly and coworkers demonstrated that the phage attacks only the streptomycin-producing strains of *Streptomyces griseus* and it has no effect on other streptomycin-producing organisms such as *S. bikiniensis*. Other species of *Streptomyces* as well as the other genera of Actinomycetes were not affected by the *Streptomyces griseus* phage. The phage multiplies only in living cells and not on the heat-killed organisms. The phage has an optimum temperature for multiplication of 28° C and it does not multiply at 37° C or above. It can withstand 75° C for one hour but it is killed at 100° C after 10 minutes. It can be stored at 6° C without loss of activity, but at 28° C or higher it soon loses its activity proportionately to the rise in temperature. The nature of the medium also affects the rate of destruction of phage activity.

Waksman, Reilly and Harris (**14**) used the phage of *Streptomyces griseus* for identifying streptomycin-producing strains of *S. griseus* especially in the fresh isolation of strains from soils.

In 1950 Koerber *et al.* (**7**) reported two different types of *Streptomyces griseus* phages. They distinguished these types by the host specificity, plaque morphology and size of particles. The larger size phage produced the smaller type of plaque.

Umezawa *et al.* (**13**) reported in 1950 their observations on the *Streptomyces griseus* phage. They found that the streptomycin yield increased for about 10 hours after almost all mycelium was lysed and the broth had become almost clear. Furthermore glucose was rapidly consumed after the broth had become clear. Among 28 streptomycin-producing strains of *Streptomyces griseus*, 17 strains were lysed by the phage. The phage was not effective against grisein-producing *Streptomyces griseus* strains, indicating that the phage was strain-specific. Therefore, they stated that the use of streptomycin-dependent bacteria for the purpose of identifying the streptomycin-producing strains of *Streptomyces griseus* was superior to the use of an Actinophage.

Perlman *et al.* (**8**) reported that the multiplication of the bac-

teriophages of *Streptomyces griseus* may be limited by the addition of substances such as citrate, oxalate and phytate capable of sequestering calcium in the media before infection of the host cells takes place. These substances inhibited phage multiplication in submerged culture fermentations without adversely affecting streptomycin production. The effectiveness of the inhibition of bacteriophage multiplication may be reversed by addition of metallic ions forming complexes with these sequestering agents.

Watson reported (**15**) that magnesium and manganese ions supported the growth of the *Streptomyces griseus* phage S-1, being required as absorption co-factors. The phage multiplication was completely inhibited by certain concentrations of the alkaline cations, sodium, potassium and ammonium. This inhibition was attributed to an antagonism of the required metals by the alkaline salts.

Abe, Shiotsu and Endo (**1**) in 1952 reported their studies on the phage of *S. griseus* S-1. They studied the morphology of the phage with the electron microscope and reported it as being of a tadpole or spermatozoidal shape with a cocoon-like head of 120–130 mμ in length, 70–80 mμ in width and a tail of 160–200 mμ in length and 30 mμ in width and called this *N-1 phage*. This Japanese strain of phage is much larger than the strain reported by Woodruff (**18**).

In 1952 Gilmour (**6**) isolated from soil a phage designated as *514-3* which was able to attack a large group of *Streptomyces* species. It was pointed out that, instead of a single kind of phage, several were probably present, as evidenced by the different morphology of the phage particles themselves and by the varied action of single plaque cultures upon the various Streptomyces species.

PHAGE OUTBREAK IN THE S. GRISEUS FERMENTATION

In March, 1952, it was observed that the mycelial growth of *S. griseus* in some fermentation tanks became suddenly and totally autolyzed. As soon as the presence of phage was suspected, it was confirmed by testing the Seitz filtrates of the broth. The end results of the phage contamination in the fermentation tanks were usually a characteristic increased fluidity, diminution or ab-

sence of mycelial growth, little or no streptomycin, the presence of a dark brown soluble pigment and the presence of the phage. The dark pigment production is due to the strong enzymatic reactions which accompany the autolytic destruction of *S. griseus* by the phage action.

Careful investigation revealed that in none of our stock culture collection of *S. griseus* was the phage present. In spite of strict sterility control measures, the infection must have been introduced in some subsequent operation during the scale-up to plant fermentation.

Through the development of phage immune strains not only was the phage eliminated, but also the streptomycin yields were significantly increased by approximately 17 per cent. Out of 111 tanks which were sampled during a three week period and tested for phage, 74 were found to be infected. Among these 74, some were also contaminated with bacteria. The remaining 47 were free of phage. All the above 111 Seitz filtrate samples were preserved at 3–5° C for further work. The fermentation samples were taken at times varying from 12–72 hours of fermentation. The amount of phage particles in the broth of these 74 infected tanks varied from 100 to approximately a trillion phage particles per ml. of the broth. This indicated that the tanks were infected at different times in the fermentation period. In testing the tank broth samples, two methods were used: 1) the whole broth was streaked on the surface of agar medium to determine type of growth of *S. griseus* and the presence or absence of contaminants such as bacteria and phage, and 2) the Seitz filtrate was tested for the presence of phage.

The first infected tank tested contained 50 billion phage particles per ml. of broth after fermenting for 42 hours at 25° C. The phage obtained from this broth was named phage No. *1*. Phages No. *1* and No. *39* were selected for experimental use throughout this work because of their highly effective mycophagy upon the *S. griseus* cultures. Phage No. *39* was obtained from broth from a 28-hour old fermentation tank.

These phages were multiplied by inoculating shake flask cultures containing the spores or young mycelial growth (24–48 hours

old) of a phage-sensitive strain of *S. griseus* with a suspension containing a few phage particles.

MATERIALS AND METHODS FOR PHAGE DETECTION

1. *Filtration of Broth Samples:* Broth samples were taken from fermentation tanks or from stationary or agitated cultures. It was necessary to separate the mycelial growth and the rough particles of media from the fermented broth by filtering first through filter paper or through cotton held between two layers of cheese cloth. The clear filtrate was then passed through the Seitz filter in order to obtain a sterile filtrate containing any phage that may have been present. These filtrations were performed with a Seitz filter open on top and connected through a well-tightened rubber stopper to a side arm pyrex flask, connected to a vacuum pump. The flask, with the filter in place, was sterilized in the autoclave previous to each filtration.

For convenience and practicability, the Seitz filters used throughout this work were the standard laboratory models commonly known as Seitz Combination Uhlenhuth-Manteufel, Laboratory Model Size 6. Once the sterile filtrates were obtained, they were tested by streaking them on double layer seed plates.

2. *Double Layer Seed Plates:* Agar plates were prepared with the following media: A or B, used interchangeably as a nutrient medium throughout this work, varying only the amount of agar to give the required percentages, or omitting it, when used as liquid media.

	Medium A	Medium B
Water	1 liter	1 liter
Dextrose	10 grams	10 grams
Difco yeast extract	10 grams	20 grams
K_2HPO_4	0.5 grams	0.5 grams
$MgSO_4 \cdot 7H_2O$		0.5 grams
Agar	15 grams	15 grams
	pH 6.8–7.0	

The agar plates (100 × 20 mm) contained a base layer of 20 ml of medium and after it solidified, a top seed layer of 2 ml was added which contained the spores of *Streptomyces griseus* (sensitive to phage), from a slant or agar plate culture. The spore suspension was added to the medium at 42–45° C just before pouring. The

bottom layer contained 2 per cent agar and the top or seed layer contained 1 per cent agar. The addition of 100 units of streptomycin per ml of medium in both layers may be used to prevent the growth of bacterial contaminants. The streptomycin was added to the upper layer at the same time as the *S. griseus* spores. This type of plate was also excellent for detecting the presence of phage in the air as described below. Before streaking the plates with the phage filtrates, the surface of the seed layer should be hardened and well-dried. Each sample can be used in three strengths:

1. undiluted filtrate
2. dilution of 1 : 10,000
3. dilution of 1 : 100,000,000

The plates were streaked with a wire loop which held 0.01 ml of sample. Three parallel streaks were made per plate in the above dilutions, care being taken not to touch the edge of the plate with the loop. The cultures were then incubated at 28° C for 1–5 days; results were observed within 16–24 hours of incubation.

One of the dilution streaks usually showed a few clear plaques. In these cases it was easy to make a rough estimate of phage particles per ml of the original filtrate. See FIG. 1.

It was interesting to note that numerous spores of *S. griseus* as well as some mycelium were often found in the areas and plaques where there was no visible growth. These spores, when transferred to fresh media, failed to germinate, implying that they were either dead cells or would be soon because of their inability to grow and multiply. These spores, when observed under the microscope, were found to be normal in size and morphology.

3. *Double Layer Phage Assay Plates:* These plates were prepared in the same way as the double layer seed plates described above, with only one difference, viz., the top layer was poured with medium containing the proper dilution of the phage in addition to the spores of *S. griseus* phage-sensitive strain. This method of using two layers of agar always gave clear-cut plaques due to the thinness and the softness of the top seed layer which was ideal for mycophagy. See plate 1 in FIG. 1. This method was superior to that in which the phage and the sensitive organisms were

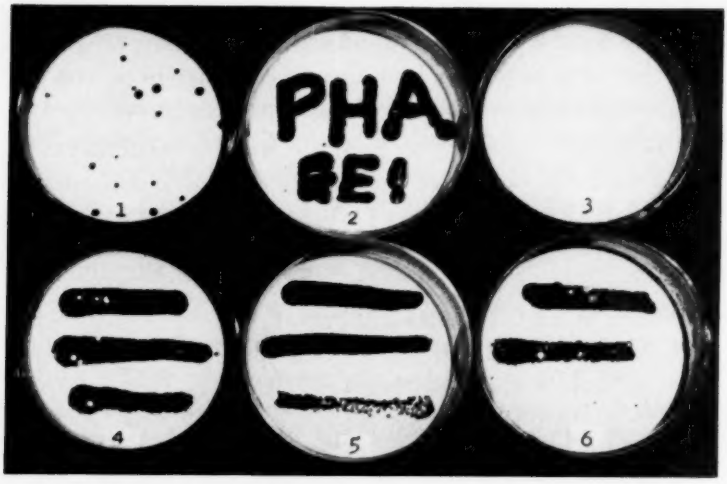

FIG. 1. Phage action upon *S. griseus U-203.* Cultures 10 days old at 25° C. Plate 1: Double layer phage assay plate showing typical phage plaques as clear circular areas without any growth of *S. griseus* while the rest has a nice heavy growth of *S. griseus.* Plate *2*: Shows the name PHAGE which was written with the aid of a wire loop on the surface of a double layer seed plate with a phage filtrate which contained 10 billion phage particles per ml. The name PHAGE is shown as clear and transparent letters without any growth among the heavy growth and sporulation of *S. griseus.* Plate 3: Control agar plate without any phage showing heavy growth and sporulation of *S. griseus U-203.* Plates 4, 5, and 6: Show the phage action from 3 different filtrates (3 different phage contaminated tanks) upon *S. griseus U-203,* in double layer seed plates. These 3 plates show clear streaks (no growth) from left to right through the *S. griseus* growth. Each streak contains 0.01 ml at the given dilution of the phage. The top clear streak in each plate is the undiluted filtrate; the second or middle streak is the filtrate diluted in distilled sterile water 1: 10,000 and the 3rd or bottom streak is the filtrate diluted 1: 100,000,000.

Note: The plaques, the moth-eaten areas, and the isolated phage-resistant *S. griseus* colonies in the phaged streaks show the typical phage action upon its host *S. griseus.* The filtrates on plates 4 and 6 gave more resistant colonies than the filtrate on plate 5.

poured onto plates in a single layer of about 15–30 ml of medium containing two per cent agar inasmuch as the plaques overlapped and resistant colonies lying between the plaques in a vertical plane obscured the results.

The phage plaques, which were the clear areas with no growth

of *S. griseus* but containing the phage, varied in size and shape (see FIG. 1, plate 1) appearing more or less round to the naked eye.

4. *Single Layer Phage Plates:* These plates were prepared by incorporating the phage into the agar medium at 42–45° C, pouring the plates and allowing them to harden. For best results, medium containing one per cent agar was used. The agar medium contained from one million to one billion phage particles per ml. The single layer phage plates were used as soon as possible to avoid drying out and consequent loss of mycophagy.

This type of plate was employed for testing cultures of *S. griseus* for phage sensitivity and phage resistance. A suspension of spores or growth of the culture was streaked on the surface and then the plates were incubated at 28° C. Results were obtained within 1–5 days.

When testing strains of *S. griseus* which produced little or no streptomycin, care was taken to avoid the presence of streptomycin in the phage filtrates because of the inhibitory action upon the growth of these inactive strains.

5. *Isolation of Phage from Air:* It was a very simple process to determine the presence of phage particles in the air of rooms, laboratories, tank fermentation rooms or out of doors. Double layer seed plates were exposed to the air for 1, 3, 5, 20, 30 minutes and 1 hour, etc., and then incubated at 28° C. Definite and clear results were quickly obtained within 1–3 days. For instance, during the short-lived phage infestation at the plant, by exposing plates to the air for 3 minutes in the fermentation tank room, 500–1000 phage plaques developed per plate. After two months no phage was obtained from the air even after long exposure, due to the employment of phage-immune strains of *S. griseus*, institution of stricter sanitation control, and the temperatures of 28–33° C usually prevailing in the fermentation rooms.

The number of plaques developing on the exposed plates was proportional to the degree of severity of the phage infestation in a given fermentation area. Repeated exposure of plates over several days or weeks gave us an idea of the effectiveness of the sanitation program. The following faulty practices led to the air becoming heavily loaded with phage particles: 1, emptying infected

tanks without first sterilizing them; 2, permitting exhaust air of contaminated tanks to be vented inside the room without the use of a trap, and 3, blowing steam through sampling valves of infected tanks after samples had been taken. The first blast of steam through the valve threw into the air a fine mist containing millions of living phage particles. The object of the use of the steam was to keep the valves clean and sterile. However, to guard against air contamination, some arrangement such as trapping should be employed. The above discussion applied equally well to other contaminants such as bacteria.

6. *Isolation of Phage from Soil:* When a particular soil was to be examined for the presence of phage, or when phage was to be isolated from soils for a particular culture the following simple procedure was followed.

Ten grams of fresh soil (or mud) were added to 100 ml of broth medium A or B which contained 100 mcg of streptomycin per ml of medium (to avoid bacterial growth), and a heavy spore suspension of *S. griseus* for which a phage was desired. The culture was incubated from 1–3 days at 28° C, the broth was Seitz filtered and the sterile filtrate was tested using double layer seed plates. Agitated cultures 24–48 hours old in medium A, B, or C on which there was a nice young mycelial growth could also be inoculated with the soil plus 100 mcg of streptomycin per ml as an optional addition. The procedure for testing followed as above.

This method can be used for testing the presence of phage not only in soil samples but in other materials such as rough media, nutrients like soybean meal, various proteinaceous and carbohydrate substances, corn steep liquor, etc.

MYCOPHAGY

The phenomenon of the mechanism of action which determines the lysis of the spores and mycelium of the fungus *S. griseus* and the formation of plaques, is dependent on the availability of free water in the medium, which helps in the diffusion of the phage and in the penetration and lysis of the cells by the phage. The water supply in liquid cultures is ideal for mycophagy, bringing the phage in contact with all the host cells. In a semi-solid medium which is

too hard, due to the presence of more than two per cent agar, the mycophagy is very much reduced or absent. Therefore it was essential to use a thinner layer and a softer agar, as for instance one per cent agar, where the water along with the phage diffused readily through the layer of medium, thus bringing the phage in contact with the host cells. The presence of free water contained in soft agar not only helped in the transportation and diffusion of the phage but also in the penetration and disintegration of the *S. griseus* cells by the phage, bringing about a more complete lysis and consequently larger and clearer plaques.

This mechanism of action can easily be demonstrated by the following examples: a) A phage filtrate was streaked at various dilutions on the surface of a double layer seed plate. The top layer of one per cent agar was seeded with spores of a sensitive strain of *S. griseus*. The phage then diffused into and through the top layer, coming into contact with the *S. griseus* spores and mycelium and penetrating them. The consequent complete lysis appeared as very clear streaks and plaques as can be clearly seen in FIG. 1. This example indicated that the available free water helped in the transportation of the phage and in the penetration and lysis of the host cells by the phage. b) From a million to a billion phage particles per ml of medium were mixed into the medium containing *S. griseus* spores before pouring plates or making slants. When adding the phage and the spores the agar is about 42–45° C. After pouring plates and making slants the cultures were incubated at 28° C for one to two weeks time. As a result, some colonies of *S. griseus* appeared to be distributed throughout the agar. Among the agar plates we found many which had numerous colonies growing around the edges of the surface of the agar where the agar was in contact with the glass walls of the petri plate. The majority of these colonies were still phage sensitive while the other colonies away from the edges of the plate were phage resistant. Among the agar slants, practically all showed heavy growth (largely phage sensitive) on the dry top portion of the slant while only a few resistant colonies appeared in the rest of the agar slant. In this example the spores and phage were brought into contact by their initial mixture in the fresh medium. c) Solidified agar slants which contained the phage

were inoculated on the surface with a heavy spore suspension of *S. griseus*. The same growth observations were noted as in example "b" above. This is illustrated by tubes 1, 2, and 3 of FIG. 3, where *S. griseus* is growing nicely on the top, drier sections of the slants, while in the center and lower portions, where moisture is more available, the mycophagy is evidenced by the growth of only a few resistant colonies of *S. griseus*. The majority of those *S. griseus* colonies which grew in the drier portions of the slant or plate were still phage sensitive, the phage being unable to bring about the phenomenon of mycophagy due mainly to lack of water.

The same phenomenon of mycophagy using *S. griseus* phage sensitive strains was also exemplified by using another fungus, *S. olivaceus* NRRL No. *B-1125*, in the presence of phage.

TESTING OF VARIOUS ACTINOMYCETES FOR PHAGE SENSITIVITY

Cultures of various species representing the five genera of the Order Actinomycetales (*Mycobacterium*, *Nocardia*, *Actinomyces*, *Streptomyces*, and *Micromonospora*) were tested for phage sensitivity, using the single layer phage plate technique.

A total of 84 cultures of *Streptomyces griseus* were used in these tests. Of these cultures 76 were isolated in our laboratories from various soils, six were kindly supplied by Dr. S. A. Waksman, Nos. *4*, *9*, *19*, *3326*, *3478*, and *3510*, and two by the Northern Regional Research Laboratory, NRRL No. *B-148* (Carvajal's *SL-2060*) and NRRL No. *B-1075*. The 84 cultures represented 66 streptomycin producers and 18 non-producers.

Of the 66 streptomycin-producing cultures only five were resistant, while the rest were sensitive to the action of the phage. Among these phage-resistant cultures, a lyophilized culture *S. griseus SL-841*, which was a good streptomycin producer, was isolated from Ohio River mud in March 1945 (**4**) and kept preserved in a lyophilized state since that date. It was interesting to notice that culture *S. griseus SL-842*, which was also a streptomycin producer and was isolated at the same time and from the same sample of soil, was very sensitive to the phage action.

All of the 18 non-streptomycin-producing cultures were not affected by the phage with the exception of the two grisein pro-

ducers, Waksman's *3478* and *3510*. Culture *3478* was more sensitive to the action of the phage than *3510*.

When a culture of *S. griseus* RH-*674*, a streptomycin producer, grew on an agar plate in the absence of phage, it grew and sporulated heavily. In the presence of the phage it gave a heavy mycelial growth showing some rosette type growth but no dry aerial sporulation.

Other species of *Streptomyces* which produced streptomycin were not affected by the phage. These cultures were kindly sent by Dr. K. B. Raper and Dr. R. G. Benedict from the Northern Regional Research Laboratories at Peoria, Illinois. The cultures were *Streptomyces griseocarneus* NRRL No. *B-1068* (Hydroxystreptomycin producer), *S. bikiniensis* NRRL No. *B-1049* (streptomycin), *Streptomyces* sp. Lilly No. *4* (streptomycin) and *Streptomyces* sp. NRRL No. *B-1350* which is Abbott No. *Na232-MI* (Hydroxystreptomycin).

Several other Actinomycetes which were tested and found to be resistant to the action of the phage were *S. carneus* ATCC-*6847*, *Streptomyces* sp. Wis. *35* (antimycin), *Streptomyces scabies* ATCC-*10246*, *S. flavus* ATCC-*3369*, *S. albus* ATCC-*618* and *3004*, *S. hygroscopicus* CBS and ATCC-*10976*, *S. roseochromogenus* ATCC-*3347*, *S. venezuelae* NRRL No. *B-902* (chloromycetin), *S. fradiae*, Waksman's *3535* (neomycin), *S. ramosus* NRRL No. *B-2334* (terramycin), *S. aureofaciens* NRRL No. *B-2209* (aureomycin), *S. olivochromogenus* ATCC-*3336* and NRRL No. *B-1341*, *S. lavendulae* (streptothricin),, *S. sulphureus* ATCC-*3007*, *Streptomyces* sp. Gottlieb (endomycin), *Streptomyces* sp. Wis. *A-158* (helixin), *S. olivaceus* ATCC-*3335* and NRRL No. *B-1224*, *Nocaria corallina* ATCC-*999*, *Nocardia asteroides* JS-*10*, *Nocardia farcinica* ATCC-*3318*, *Actinomyces bovis*, *Micromonospora* sp. ATCC-*10026*, *Mycobacterium tuberculosis*, *M. phlei*, *M. smegmatis*.

On the other hand not only *S. griseus* but also *S. olivaceus* NRRL No. *B-1125*, *S. griseolus* ATCC-*3325*, and *S. viridis* ATCC-*3372* were susceptible to the action of the *S. griseus* phage. With these cultures and using pure cultures of phage, typical plaques of phage were produced as well as the lysis of the spores and mycelium with the consequent multiplication of the phage particles.

These facts clearly demonstrated the polyvalent nature of the *S. griseus* phage which was able to attack and lyse four or more different species of *Streptomyces*.

Two main and strikingly different types of resistant colonies were found among the surviving population of *Streptomyces olivaceus* NRRL No. *B-1125* after the phage attack; one was a new type, sulfur-yellow in color, and the other olive-colored, like the parent culture. The growth characteristics in streaked plates noted after five days' growth and thereafter of these two colony types in agar media A and B at 25° C and at 28° C, can be described as follows:

1. *Sulfur-yellow Colonies:* The most numerous ones constituting about 60 to 90 per cent of the total of phage-resistant colonies. Surface growth abundant, little spreading, very finely corrugated or folded throughout, giving a spongy to rosette-like appearance. Top and the reverse are of the same sulfur-yellow coloration. A faint yellow-soluble pigment is produced by these colony cultures. In medium B the growth and color are more intense than in Medium A. The growth is scraped off easily and in distilled water gives a granular suspension; under the microscope the mycelium is sulfur-yellow; it contains practically no spores or very few. The growth of these colonies, in general, resembles very much that of the various species of the genus *Mycobacterium*.

2. *Regular, Olive Color, Colonies:* They constitute about 10 to 40 per cent. These colonies are of the same appearance as the parent culture which never was exposed to phage. The surface growth is abundant, spreading, developing deeper into the medium than the sulfur-yellow colonies; smooth appearance with a few folds especially at the edges of growth; very heavy aerial sporulation of a light to dark olive color of a powdery appearance; reverse of colony from a dark olive color to an almost black. A faint olive colored soluble pigment diffuses out into the agar medium.

It was of interest to note that *S. griseus* and *S. olivaceus*, which were two of the best microorganisms used in the commercial production of vitamin B_{12}, were both affected by the same phage. Pure cultures of phage derived from single plaques developed in *S. griseus* cultures were able to attack *S. griseus* and *S. olivaceus*, and single plaques from *S. olivaceus* were able to destroy *S. griseus* as well as *S. olivaceus*. Single spore isolations of *S. olivaceus* were made with the aid of a micromanipulator and the cultures after de-

velopment were tested and found to be as sensitive to the action of the phage as their parent culture.

DEVELOPMENT OF IMMUNE AND RESISTANT COLONIES OF S. GRISEUS

The most important measure in phage control is the development of an immune or a highly resistant strain of *Streptomyces griseus* to the attack of the virus. The different cultures of *S. griseus* show various degrees of resistance to a given phage.

SINGLE COLONY ISOLATION: Resistant colonies of *S. griseus* were easily obtained from agar or liquid media cultures grown in the presence of the phage. The addition of 100 mcg of streptomycin per ml of medium helped in the inhibition of *S. griseus* strains which were low or non-streptomycin producers. The presence of streptomycin did not interfere at all with phage growth. In over 200 single colony cultures tested, it was shown that the phage grew and multiplied equally well, and sometime better, in the presence of 100 mcg of added streptomycin per ml of medium as compared with medium without added streptomycin.

A. From Solid Media. Medium A or B, or any other suitable one, containing the phage (one million to a billion phage particles per ml) and a heavy spore suspension of *S. griseus*, sensitive to phage, was poured out and incubated at 25–28° C for 1–2 weeks after which time colonies were isolated. Agar plates containing the phage were streaked on the surface with the sensitive strain of *S. griseus*, and later on the resistant colonies which grew were isolated.

B. From Agitated Cultures. The production of phage resistant strains of *S. griseus* can be accomplished using medium A in shake flasks. If a defoamer is desired, lard oil can be used at 0.1 ml per 100 ml of medium. The shake flasks were sterilized at 120° C and 15 lbs pressure for 20 minutes and each flask (liter capacity) contained 200 ml of medium. The shaker was set at about 92 reciprocating strokes per minute. Each flask was inoculated at the same time with a potent phage filtrate and with a heavy spore suspension of a currently used strain of *S. griseus* sensitive to phage. One hundred micrograms of streptomycin per ml. of medium was added in this first step. The flasks were placed on the shaker at 25° C

for 24–48 hours. A pelleted type of growth resulted. These clearly conspicuous (mycelial growth) pellets were the phage-resistant colonies of *S. griseus,* most of which originated from a single spore. It is important to point out that agitated cultures should not be run over 48 hours because the mycelium will start fragmenting and the culture will deteriorate, making it undesirable for single colony isolation.

The pellets from the flasks were then plated out in agar medium A containing 100 mcg of streptomycin per ml to grow and sporulate, for the single colony isolation. About 0.1 to 1 ml of broth from infected flasks containing the phage and the pellets was used to inoculate each 100 ml of agar medium at 42–45° C. prior to pouring the plates. About 10–15 ml were used per petri plate. After the agar had solidified the plates were incubated at 25–28° C for 1–2 weeks before colony selection was made.

Using the above procedure it was possible to isolate several colonies of *S. griseus* per plate, showing varying degrees of resistance and even immunity to phage. Well developed healthy and sporulated colonies were selected for isolation to agar slants of medium A or B. The agar slants contained the phage and 50 mcg of streptomycin per ml. The slants were grown for 7–10 days at 25–28° C and then tested in medium C in shake flasks for streptomycin and B_{12} producing abilities. The best cultures selected did not require the incorporation of any more phage in the medium in successive transfers.

MASS COLONY RESISTANCE: The mass colony resistance from submerged cultures or from agar media usually produced lower yields. Several experiments were run by developing good mycelial growth in shake flasks within 24–48 hours from heavily inoculated cultures with spores of a phage-sensitive strain of *S. griseus,* to which phage was added at the same time. This submerged inoculum, from 5 to 20 per cent by volume, was used to inoculate fresh medium. As a result the streptomycin yields were low. When this phage-mass-resistant mycelium was used to inoculate agar media A or B, or any other media, good mycelial growth was obtained but aerial (buffish) sporulation was very scanty. For details see plate 3 in FIG. 2 and tube 4 in FIG. 3. This was in contrast to the phage-sensitive strain (never exposed to phage) which always

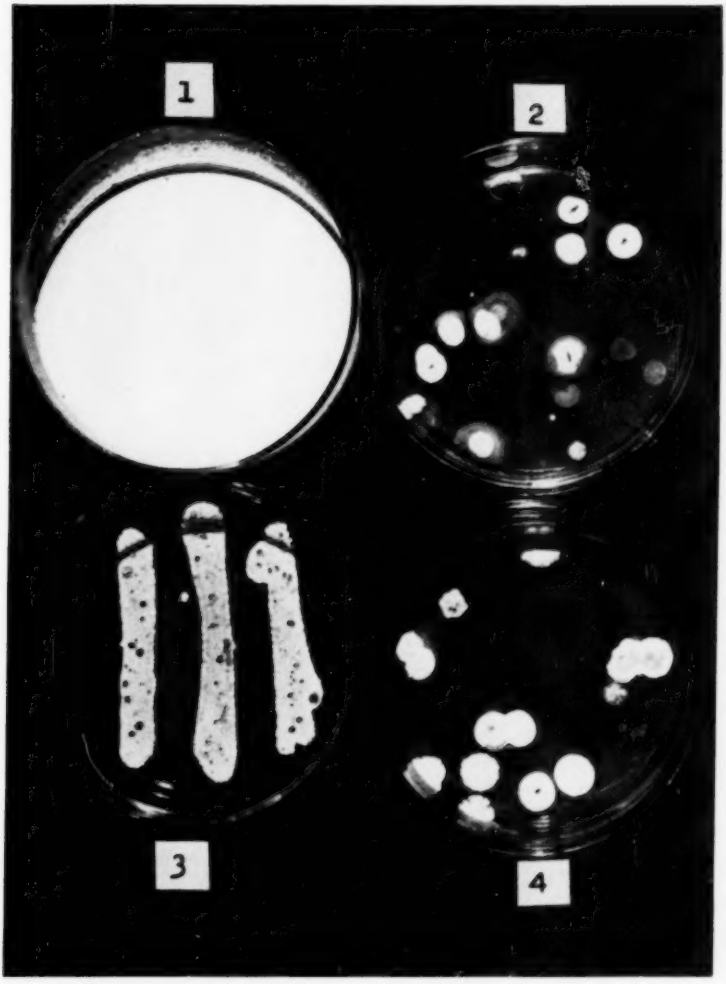

FIG. 2. Phage action upon *S. griseus U-203* cultures 3 weeks old at 25° C. Plate 1: Control plate seeded with *S. griseus U-203* without phage; it shows heavy growth and sporulation. Plates 2 and 4: Seeded, as plate 1, with the same amount of spores of *S. griseus U-203* but adding phage (100 million particles per ml). Note the different types of resistant colonies which developed. Plate 3: Shows three streaks of growth. Single layer phage plate was inoculated with the 3rd generation through submerged liquid culture of a phage mass resistant culture. It shows poor sporulation and numerous yeasty (rosette) colony types throughout each streak.

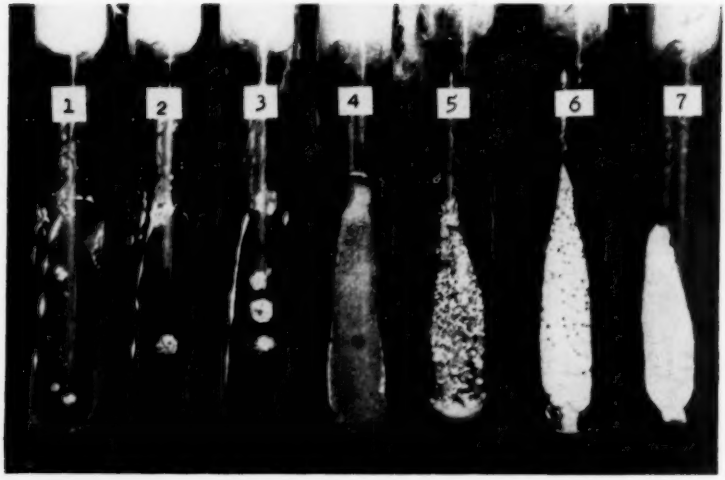

FIG. 3. Phage action upon *S. griseus U-203* cultures 10 days old at 25° C. Various colony types. All the agar slants contained the phage, 100 million phage particles per ml. Tubes 1, 2, and 3: These agar slants which contained the phage were heavily seeded on the surface with phage sensitive culture of *S. griseus U-203*. The various colony types which are shown growing here are the phage-resistant ones. Tube No. 2 contains a typical granular type colony. Tube No. 3 shows a yeasty (rosette) type colony of a nice size toward the bottom of the slant. Tube 4: Inoculated with the mycelium of a submerged third generation of a mass colony (mycelium) resistant culture. Note the poor sporulation, the phage plaques, the varied types of growth and a large yeasty colony type growth toward the center of the slant. Tube 5: Inoculated with spores from a resistant colony obtained from an agar plate. Note the rise of various colony types and the general phage action. Tube 6: Contains a nice resistant colony culture which still is attacked by the phage (fourth generation) as is shown by the number of phage plaques. Tube 7: Shows a heavy sporulation and phage-immune culture of *S. griseus*.

sporulated heavily on agar medium even after passing 12 transfers (every 24 hours) through submerged mycelial growth. In both cases the streptomycin yields were greatly lowered by the end of the twelfth submerged transfer.

S. GRISEUS COLONY TYPES RESULTING AFTER PHAGE ATTACK

FERMENTATION: The action of phage on a sensitive culture of *S. griseus* brought about a variety of colony types which were

strikingly different in morphological, cultural, and biochemical characteristics. These varying types among the surviving population were comparable to those mutants produced by the action of ultraviolet light, X-rays, and nitrogen mustard gas. Therefore, we are making use of this biological principle in our mutation or selection program.

In the mutation work of *S. griseus* for streptomycin and B_{12} production, phage can be used alone, or in combination with one or more of the other agents for selection and mutation such as streptomycin, ultraviolet light irradiation, X-rays, nitrogen mustard gas, or any other mutagenic agent.

When agar dilution plates were made of spores of a phage-sensitive *S. griseus* culture plus phage, with or without streptomycin, five main colony types were developed within one to three weeks, as follows:

1. *Pin Point Type Colonies:* Pin point or very small colonies on the surface or submerged in the medium, mostly without sporulation and hard; some very soft; with little or no color.
2. *Poor Sporulation Type Colonies:* Flat, thin, strongly adhered to the agar, usually of a fairly large size. Often dark in color, with or without dark soluble pigment; some colonies almost colorless.
3. *Regular or Normal Type Colonies:* Typical *S. griseus* well-developed colonies, powdery, of buff to olive greenish color aerial sporulation. This is the type preferred for isolation.

These colonies of the regular or normal type showed varying degrees of resistance as described below:

Nice growth and sporulation was seen after plates were incubated from one to three weeks. By looking at them closely with the naked eye one can easily see their sporulation types. Most of the colonies appeared to have chewed-up or moth-eaten areas in various patterns, on the top or at their edges.

The surface of a single colony may show a few or several large and small areas with varying degrees of sporulation from heavy to almost naked. Some details described here can be seen in FIG. 2, plates 2 and 4. When sub-cultures from the spores of these colonies (see FIG. 3, tubes 5 and 6) were made to agar slants, they usually had from 1 to 100 phage plaques and sometimes more, so

as to be almost completely without sporulation, just mycelial growth. The phage-resistant cultures of poor sporulation or with many plaques were usually poor streptomycin producers, and the results obtained were very inconsistent. Some of these cultures, which were fairly good producers of streptomycin but still carried the phage, were a menace because new strains of phage which attacked and destroyed this culture were produced during fermentation.

Immune Cultures: Among the resistant colonies, there were a few with a nice growth, exhibiting smooth and complete sporulation without any moth-eaten areas. These colonies, upon transfer, usually produced perfect cultures without plaques and without moth-eaten areas (FIG. 3, tube 7). Some of these cultures were immune to the phage action; they did not produce any plaques even when large amounts of phage were added and the phage failed to multiply in these cultures growing in agar or in liquid cultures. After 20 generations, they were still phage-immune, as shown by various tests performed. Furthermore, no phage was recovered after complete autolysis of the mycelium from submerged cultures. This was the ideal type of culture. The cultures chosen for commercial

TABLE I

RESULTS OF 130 REGULAR TYPE CULTURES TESTED IN SHAKE FLASKS. MEDIUM C[1]

Number of cultures	Range of streptomycin production mcg per ml
1	None
42	200–600
4	600–800
25	800–1000
40	1000–1400
11	1400–1600
5	1600–1800
2	1900–2200

[1] *Shake Flask Medium C:*

Distilled or tap water	1 liter
Soybean meal, 4S (Staley's)	20 grams
Corn steep liquor	10 grams
Dextrose (or cerelose)	10 grams
Dextrin	10 grams
$MgSO_4 \cdot 7H_2O$	3 grams
Cobalt (as chloride, sulfate or nitrate)	0.25 ppm

pH adjusted to 7.5 with NaOH before sterilization (after sterilization pH 6.5)
Volume 200 ml per liter flask
Lard Oil 0.2 ml per flask
Sterilization 20 minutes at 120° C, and at 15 lbs pressure.

TABLE II

RESULTS OF 50 GRANULAR TYPE CULTURES TESTED IN SHAKE FLASKS. MEDIUM C

Number of cultures	Range of streptomycin production mcg per ml	Range of B_{12} production mcg per liter
6	None	147–710
17	10–25	119–295
16	26–50	109–228
4	55–80	176–218
2	200–300	96–200
2	400–500	109–130
3	600–700	141–320
Control (parent culture)	1100	400

production were of this immune type. The final choice was made from those cultures of this group which showed consistently high production of streptomycin and B_{12}.

TABLE I shows the results in shake flask fermentations of 130 cultures of the regular type colonies described above.

The control, parent culture *S. griseus U-203*, produced from 1000 to 1400 mcg of streptomycin per ml of medium C; and about 400 mcg per liter of B_{12}. The pH of the broth rose within 1 to 7 days from 6.5 to 8.2–8.6. All the cultures grew very well, and the pH changes as well as the B_{12} yields were very similar to the control culture. The greatest difference was in streptomycin production, in which about 12 cultures out of 130 tested in this experiment proved to be superior to the parent culture *U-203* (phage sensitive). All the regular type cultures seem to be more proteolytic than the other colony types tested here. These excellent results were obtained by treating culture *S. griseus U-203* with phage No. 1. But treatment of another culture, *S. griseus U-203-09*, in the same way but with another phage, isolated No. 39, failed to produce a single superior streptomycin producing strain among 400 strains so tested.

4. *Granular Type Colonies:* Colonies of granular appearance and consistency vary from very small to large and are much raised—rugose. These colonies are usually of a slight pink coloration; appearing from about 0.1 to 1% of the total of colonies. They are of a hard consistency and make a granular suspension (see FIG. 3, tube 2).

In TABLE II the shake flask fermentation results obtained with 50 granular type cultures are summarized.

The type growth and the pH behavior of the granular cultures in shake flasks were very much the same as the parent control cultures but all the granular cultures, without exception, gave inferior streptomycin yields. All the cultures produced B_{12}, but less than the parent culture, with only two exceptions. Culture *354* produced 620 mcg and culture *355* produced *710* mcg of B_{12} per liter of broth; however, these two cultures did not produce any streptomycin.

5. *Yeasty Type Colonies—Rosette:* Yeast-like type colonies appear wet, like a culture of yeast or bacteria, very soft; some are mucilaginous. They appear with a frequency of about 0.1 to 5%. They may appear as flat or as wrinkled or in donut and rosette shapes (see FIG. 2, plate 3 and FIG. 3, tubes 3 and 4). The color varies from dirty white to slight yellowish. These colonies are scraped off easily and all of them make a nice and homogeneous cloudy cell suspension. Microscopically they have regular size spores and some cultures have a few very large spores from 1 to 3 microns and even more in diameter resembling sporangia of some Phycomycetes; the mycelium usually short, in fragments, very often continuous and worm-like or with little ramification, or bearing spores singly or in chains. Most of these strains seem to be lysogenic. Most of these cultures produce a dark, sometimes almost black, soluble pigment in agar medium and liquid medium. A few produce no soluble pigment. In shake flasks they grow and sporulate (submerged) like the parent culture; but mycelium often in shorter threads; some cultures had very thin mycelium, some of larger dimensions, some straight, some very wavy and coarse, some long and very branched, some very short, when compared with parent culture (never treated with phage).

A total of 50 different cultures of these types were tested in shake flasks for growth, streptomycin, and B_{12} production in *medium C* and the results are tabulated in TABLE III.

All the above 50 cultures produced vitamin B_{12}, and produced it at an earlier date than the control parent culture. The pH increased from 6.5 to 8.0 within 1 to 8 days more slowly than in the control culture. As a general rule, in shake flask fermentation these cultures grew more slowly than the control and the growth of most of them was never as profuse.

With reference to streptomycin production, out of the 50 cultures only nine produced some streptomycin. But of these nine

TABLE III

RESULTS OF 50 YEASTY TYPE CULTURES TESTED IN SHAKE FLASKS. MEDIUM C

Number of cultures	Range of B_{12} production mcg per liter
28	301–400
9	401–500
5	501–600
4	601–700
3	701–800
1	901–1000
Control (parent culture)	400

cultures, eight produced only from 15–30 mcg of streptomycin per ml of broth and from 300–400 mcg of B_{12} per liter of broth. The ninth produced 270 mcg of streptomycin and 700 mcg of B_{12} per liter of broth. The other 41 cultures produced no streptomycin, all the high B_{12} producers being among them.

PRESERVATION OF HIGHLY PRODUCTIVE CULTURES OF S. GRISEUS

Once a culture of *S. griseus* has proved to be an excellent streptomycin producer, enough spore inoculum should be preserved. It should be stated here that a good culture should not be passed through many generations before commercial production of streptomycin, because many of the cultures tend to reduce their streptomycin productivity. The lyophilization of the cultures is the best insurance of maintaining a highly productive culture. Therefore enough ampules of desirable size are lyophilized in order to have enough initial inoculum to be used periodically over a period of years. At present, we have three different cultures of *S. griseus* which were lyophilized 8 years ago, and have maintained their biochemical and cultural characteristics identically with the parent cultures of 8 years ago. The lyophilization procedure used here for nearly ten years was described by Wickerham and Andreasen (**16**) and by Carvajal (**4**) using Difco dehydrated skimmed milk. For streptomycin production of lyophilized culture is opened periodically, *e.g.*, each month, and Fernbach's containing agar medium B plus 100 mcg of streptomycin per ml are surface inoculated (2 ml spore suspension per flask) and grown at 25° C for 7–10 days, then used, or stored at 3–5° C until used. The spores of each Fern-

bach are used in suspension to inoculate the seed tanks directly (one Fernbach for each seed tank of 300–500 gallons). The young vegetative inoculum from the seed tanks is used to inoculate the fermenters for the production of streptomycin.

Complementary to the lyophilization of the cultures, *S. griseus* is also kept in soil cultures, cultures under mineral oil, and the regular agar slant cultures (for slants, large tubes 6 inches high and 1 inch in diameter are preferred).

For the soil cultures, spores straight from agar cultures are incorporated into fairly dry garden soil which has been previously sterilized in the autoclave. These soil cultures are immediately stored at 3–5° C. This method has given better results than the usual method (**11**) of inoculating the soil with a spore suspension and permitting the organism to grow and reproduce for one week or so prior to storage at 3–5° C.

PRESERVATION OF PHAGE

1. *Stock Collection of Phage Filtrates:* It is very important to keep the broth Seitz filtrate of every infected tank. By this means one can detect new strains of phage which normally are produced, and thus be protected against them. These filtrates can be used singly, or in composite samples, in the development and selection of resistant and immune strains of *S. griseus* and in the selection of superior streptomycin and B_{12} producers.

2. *Lyophil Preservation of the S. griseus Phage:* The drying technique used here is essentially the same as that described in 1942 by Bickerham and Andreasen (**16**) for yeast. The writer used two different steps in the lyophilization of the phage:

A. The suspension of the phage particles in Difco skimmed milk, blood serum or any other protein rich medium.
B. The suspension of the phage as in A, plus fresh spores of a phage-sensitive strain of *S. griseus*.

These suspensions are immediately lyophilized. Strains of phage lyophilized in skimmed milk as in A above and kept at room temperatures for a period of two months have been found to retain their initial physiological and biochemical characteristics.

SUMMARY

Effective measures for the control and eradication of the phage contamination depend upon strict sanitary precautions throughout the laboratories and fermentation areas and mainly upon the development of cultures immune to the action of the phage.

The end result of the virus infection in the fermentation tanks is usually a characteristic loss of viscosity, diminution or absence of mycelial growth, little or no streptomycin, the presence of a dark brown soluble pigment and the presence of the phage.

The action of phage in a sensitive culture of *S. griseus* brings about a variety of colony types which are strikingly different in morphological, cultural, and biochemical characteristics. These varying types among the surviving population are comparable to mutants produced by the action of ultraviolet light, X-rays, and nitrogen mustard gas. With the development of phage-immune strains of *S. griseus*, not only was the phage eliminated but also the streptomycin yields were significantly increased.

Of the various Actinomycetes cultures tested, strains of *Streptomyces olivaceus*, *S. griseolus*, and *S. viridis* were sensitive to the phage as well as *S. griseus*. Therefore, the *S. griseus* phage is of a polyvalent nature, being able to attack and lyse four or more different species of Streptomyces.

The mechanism of action which determines the lysis of the spores and mycelium of the fungus *S. griseus* and the formation of plaques is dependent on the availability of free water in the medium which will help in the diffusion of the phage and in the penetration and lysis of the cells by the phage.[2]

The writer wishes to acknowledge appreciation for the assistance of Mr. Ralph Hill, Mrs. Anna Mae Cottingham, Mrs. Mary Spargo, and Mrs. Verna Schulenberg, to Mr. James Burns who performed all the streptomycin and vitamin B_{12} assays, and to Mr. Gabor B. Levy for the photographic work.

[2] After the manuscript for the present paper had been completed, a publication by J. Bronfenbrenner appeared in Science **116**: 517, November 14, 1952. Some of the observations made by Dr. Bronfenbrenner may be explained by the same phenomena.

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SIZE IN RELATION TO THE RATE OF MIGRATION IN THE SLIME MOLD DICTYOSTELIUM DISCOIDEUM [1]

JOHN TYLER BONNER, PAUL G. KOONTZ JR., AND DAVID PATON

(WITH 2 FIGURES)

It is characteristic of the Acrasiales or amoeboid slime molds that a group of uninucleate amoebae aggregate, but in only one species described thus far, *Dictyostelium discoideum* Raper, does this cell-aggregate or pseudoplasmodium migrate for a period of time before the final fruiting or culmination. The migrating pseudoplasmodium has a sausage or cartridge shape and moves slowly over the substratum secreting a slime sheath that is deposited as a track posteriorly. It has been studied by a number of investigators [2] and found to have numerous interesting structural and physiological properties, although many aspects of its behavior remain unexplained, including the mechanism of its motion. In order to collect more information concerning this interesting stage of development, a study has been made of the rates of movement and it was found that similar to the rates of culmination (3) the larger the pseudoplasmodium, the greater the speed of migration. As will be shown, this lends evidence to the hypothesis that the propelling force for movement is internal and depends on the total mass of cells rather than a superficial cell layer.

METHODS

The slime mold was grown in a two membered culture with *Escherichia coli* on the same nutrient medium used in previous studies (2). Prior to migration, when the cells had just assembled

[1] This work was carried out with the help of a grant from the American Cancer Society and with funds of the Eugene Higgins Trust allocated to Princeton University.

[2] See Raper (6) for references to the work of Raper, Gregg and Bonner. Also there is some more recent work of Slifkin and Bonner (7).

into pseudoplasmodia, certain of these were transferred with a steel spatula to the surface of a non-nutrient, 2% agar medium. As has been shown by Slifkin and Bonner (**7**) this medium favors prolonged migration, thereby facilitating prolonged observations on the same pseudoplasmodium. During both the growth and the subsequent migration stages the petri dishes were kept at 20.5 ± 0.5° C. (in the dark) and during the migration period the dishes were kept within a closed moist chamber to prevent desiccation.

As soon as migration started a camera lucida drawing was made of the pseudoplasmodium, and this was repeated at approximately 12- or 24-hour intervals. The distance travelled was traced (by following the slime track) with india ink on the bottom of the petri dish. This was later measured by placing the petri dish bottom in a lantern slide projector and tracing the projected track on the screen with a map mileage indicator and making the necessary unit conversions. The volume of the pseudoplasmodia was estimated by the segment method given in Bonner and Slifkin (**4**). Since

TABLE I

SHOWING THE RELATION BETWEEN THE VOLUME OF MIGRATING PSEUDOPLASMODIA AND THEIR RATE

(The volumes are a calculated mean of the volume before and after a given period of migration for which the rate has been determined.)

Pseudoplasmodium number		Volume in mm.³	Rate in mm./hr.	Pseudoplasmodium number		Volume in mm.³	Rate in mm./hr.
1	1	.0438	2.00	5	1	.0168	.92
	2	.0400	1.75		2	.0153	.59
	3	.0350	1.25		3	.0129	.33
					4	.0103	.25
2	1	.0337	1.25				
	2	.0238	1.04	6	1	.0236	1.63
	3	.0177	.83		2	.0209	1.14
	4	.0163	.79		3	.0195	.80
					4	.153	.70
3	1	.0259	1.61		5	.0126	.68
	2	.0224	1.16				
	3	.0155	1.00	7	1	.0414	1.26
	4	.0118	.76		2	.0399	1.17
	5	.0101	.72		3	.0315	.47
	6	.0064	.61		4	.0247	.48
	7	.0048	.53				
				8	1	.0437	1.33
4	1	.0412	1.50		2	.0363	1.07
	2	.0396	1.33		3	.0255	1.01
	3	.0337	.95		4	.0217	.63
	4	.0245	.91				

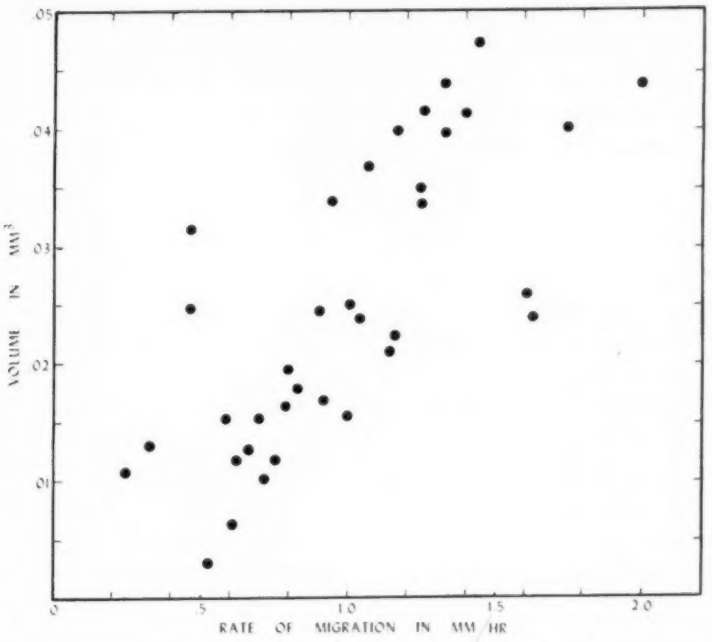

FIG. 1. Graph showing the relation of the volume of migrating pseudoplasmodia (ordinate) to their rate of movement (abscissa).

the rate is given by distance between two observation points (camera lucida drawings) in a given time, the volume equivalent to this rate was estimated by obtaining the mean volume of the two drawings.

RESULTS AND DISCUSSION

The results for eight different migrating pseudoplasmodia are given in TABLE I, and if each average volume is plotted against the rate, it is obvious that the larger the volume, the greater the rate (FIG. 1). This fact is even more clearly seen in an individual pseudoplasmodium, for as migration proceeds the pseudoplasmodium becomes smaller, and its speed is correspondingly slower (see TABLE I and FIG. 2). The reason for the decrease in size during migration is probably two-fold: some of the cells straggle and are lost in the slime track, and also the pseudoplasmodia expend energy without taking in food.

These results parallel previous work on culmination (Bonner and Eldredge, 3), where again the larger the pseudoplasmodium the greater the rate of upward rise into the air. As was pointed out then, the results on culmination, and now we may include the ones given here on migration, fit in with the hypothesis involving the

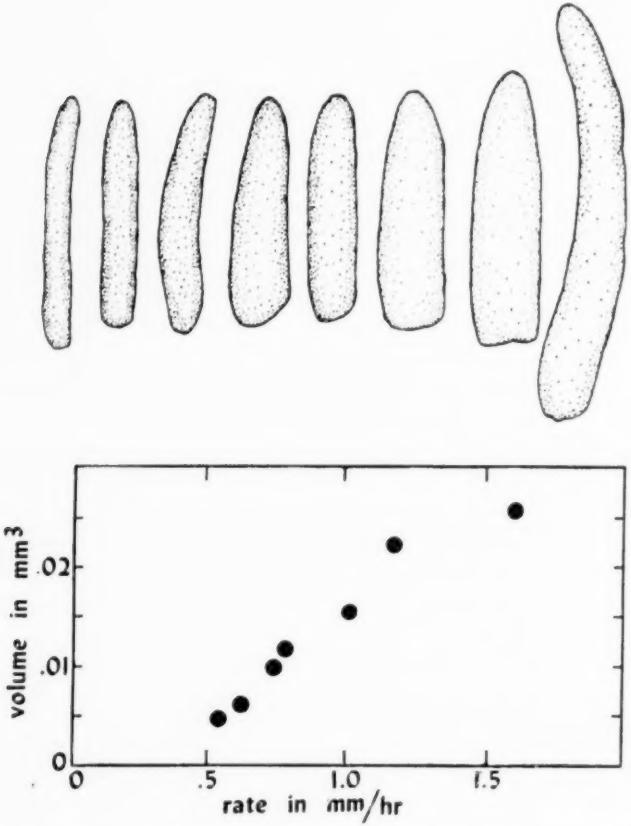

FIG. 2. *Above.* A series of camera lucida drawings of a single pseudoplasmodium (No. 3 in TABLE 1) showing, from right to left, the gradual decrease in size upon migration. *Below.* A graph of the same pseudoplasmodium in which the ordinate is the volume and the abscissa the rate of movement. (The volume values are each a mean between two of the camera lucida drawings above, corresponding to a particular rate measurement.)

principle of similitude originally proposed by Tyler (**8**) for a somewhat similar situation in sea urchin embryos.

According to the principle of similitude, in similarly shaped bodies of different size, the volume of surface ratio will increase with size, for volume increases as the cube of the linear dimensions, but the surface as the square. Since large migrating pseudoplasmodia move faster, the locus of the movement force is likely to be dependent on the volume or mass rather than the surface. On the other hand the resistance to movement or friction could be a surface phenomenon. This hypothesis assumes that the power of movement per unit mass of cells is constant for different size pseudoplasmodia, and Gregg (**5**) has shown that this assumption is supported by the fact that the oxygen consumption per unit nitrogen of tissue is constant irrespective of size.

There has been much speculation concerning the nature of this migration movement and relatively few facts. It is known that the internal amoebae are actively pseudopodial and that the individual amoebae may, to some degree, travel at different rates (Bonner, **1**). We may now postulate from the evidence presented here, that all the amoebae are actively involved in the forward progression and somehow gain traction from both the slime sheath they secrete and from one another below the surface of the cell mask.[3] The idea that the forward progression is achieved solely by the outside, superficial layer of cells is not supported by the present evidence.

SUMMARY

In the amoeboid slime mold *Dictyostelium discoideum*, separate amoebae aggregate to form sausage-shaped migrating pseudoplasmodia. The rates of movement of these pseudoplasmodia were found to be proportional to their size. This was true even of indi-

[3] There is no objection to the idea that an internal amoeba might obtain traction from his neighbors provided there is an anchor (the slime sheath) somewhere in the system. An amoeba moves by keeping its sides stiff, in a gelled condition, while the internal fluid protoplasm spouts forward at the anterior tip. If the lateral stiff portions of each amoeba stick to one another they will automatically have their traction. Also it is conceivable that each amoeba gives off slime which has some rigidity for traction and is ultimately deposited inside the slime track.

vidual pseudoplasmodia, for the longer one migrated the smaller it became and its rate decreased correspondingly. An hypothesis was presented, based on the principle of similitude, to the effect that migration movement was dependent on the total mass of amoebae, and not upon a superficial layer of specialized locomotory amoebae.

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DETECTION OF HISTOPLASMA CAPSULATUM AND OTHER FUNGUS SPORES IN THE ENVIRONMENT BY MEANS OF THE MEMBRANE FILTER

MORRIS A. GORDON AND HORACE B. CUPP, JR.

(WITH 2 FIGURES)

The quantitative determination of fungi in the physical environment usually is undertaken by means of cultural procedures, most commonly by exposure of suitable media in Petri dishes with subsequent counting and identification of colonies. For those organisms possessing diagnostic microscopic structures, more rapid detection may be accomplished by the simpler method of examining petrolatum-coated gravity slides or similar devices (**6**). Among the fungi particularly suited to direct examination methods is the human pathogen, *Histoplasma capsulatum*. At the time the present studies were undertaken, this organism had not been found to occur naturally in air or water, but had been recovered from soil on several occasions (**1**, **3**, **5**). In all of these successful attempts the technique employed was essentially that of Stewart and Meyer (**11**), involving inoculation of a soil suspension intraperitoneally into mice and subsequent recovery of the fungus by culture of various tissues. Emmons was able also to demonstrate directly, in some of his soil suspensions, characteristic tuberculate spores of *H. capsulatum*. Grayston, Loosli, and Alexander (**5**), in obtaining the fungus repeatedly from organic debris in a silo to which human infection had been traced, gave strong support to the popular concept that histoplasmosis is a dust-borne infection. It appears likely, also, that the etiologic agent may be ingested with food or water. In order to aid in the further exploration of these possibilities, and to find a rapid and efficient method for the detection in the environment of fungi in general, the use of the Goetz membrane has been investigated.

The membrane filter, which permits rapid passage of large volumes of air and water while retaining particles the size of bacteria and smaller, is admirably adaptable to the detection of fungus spores in these media. Solid materials such as soils may also be searched with the aid of flotation techniques. The history of the membrane, and accounts of its employment in sanitary bacteriology, are provided in recent papers by Paccagnella (**10**) and Clark *et al.* (**2**). These workers cultured the exposed membranes on absorbent pads saturated with selective and differential media for the isolation of bacterial colonies. Fungi also may be cultured in this manner, but more rapid detection is made possible by direct examination of stained membranes for characteristic spores.

For the latter purpose there has been devised a staining procedure which permits excellent differentiation of fungus structures from other debris as well as from the homogeneous background of the filter disc. Advantages of this filtration and staining procedure over previous methods, direct or cultural, include the following:

1. More rapid detection of fungi. Following filtration, the membrane may be processed and examined thoroughly within an hour.
2. Large sample volume, limited only by the amount of debris in the material being filtered.
3. Efficiency. *All* of the spores in the sample remain on filter discs of suitable porosity.
4. Simplicity of apparatus. Culture media and incubators are not needed.
5. The method is applicable equally to the examination of gases, liquids, and solids.
6. As contrasted with cultural methods, there is no dependence upon the ability of the fungus to outgrow other organisms or to overcome inhibition.
7. It is especially suitable for the rapid detection of gross contamination of the environment.

The chief limitation of this method is the necessity for identifying fungi solely on the basis of microscopic structure. In the case of *H. capsulatum*, the spores seem to be sufficiently distinct to minimize this deficiency.

It may be pointed out that the apparatus and techniques to be described are quite suitable for the counting of pollen grains, which take the stain very well. An additional application, omitting the stain, came to our notice in the course of this work. Legler (**9**) has employed the membrane filter for counting *Ascaris* eggs in sewage effluent and feces.

APPARATUS

The first filter membranes employed in these experiments were prepared in Dr. Goetz' laboratories at the California Institute of Technology. Subsequent discs ("Millipore Filters, Type HA" [1]) were purchased from Lovell Chemical Co.,[1] Watertown, Massachusetts. All were 50 mm in diameter and of unspecified porosity. The hydrosol filtration apparatus (FIG. 1), designed by Dr. Goetz and obtained from the L. R. Burt Co.,[1] Arcadia, California, is essentially identical with that employed by Clark *et al.* (**2**), consisting of a stainless steel funnel of one liter capacity connected by a bayonet locking nut to a receptacle containing a porous plate. The filter disc is locked on top of the porous plate, through which the filtrate drains into a filter flask.

Suction was obtained by means of either an electric vacuum pump or an aspirator operated on water pressure from a tap. Inserted in the line between the aspirator and the filter flask were a one-liter flask to serve as a water-trap and a side-arm with screw clamp for regulating the pressure. In field operations where water-pressure lines and vacuum pumps are not available, sufficient suction may be obtained by utilization of the exhaust manifold of a motor vehicle.

Although inferior in some respects to the filter membrane for direct examination, certain grades of filter paper, having the advantages of more ready availability and less expense, may also be employed for the retention of fungus spores. Whatman filter papers No. 3 and No. 50 have served successfully when fitted in Seitz filters of 30 ml. or 100 ml. capacity. The filtration speed of the No. 50 disc approximated that of the membrane, while No. 3 was much more rapid. All three serve equally well for cultural

[1] Trade names and commercial sources are mentioned as a means of identifying the products under discussion, and their use is not intended to represent endorsement of the products by the United States Public Health Service.

methods, but the Goetz membrane has the advantage of optical homogeneity for direct microscopic examination. However, the smooth surface of the membrane permits trapped spores to be washed off more readily during the staining process. It is possible also, in employing membranes, to substitute Seitz apparatus of suitable size for the more specialized filter equipment. With such an arrangement a reinforcing disc of blotting paper must be placed

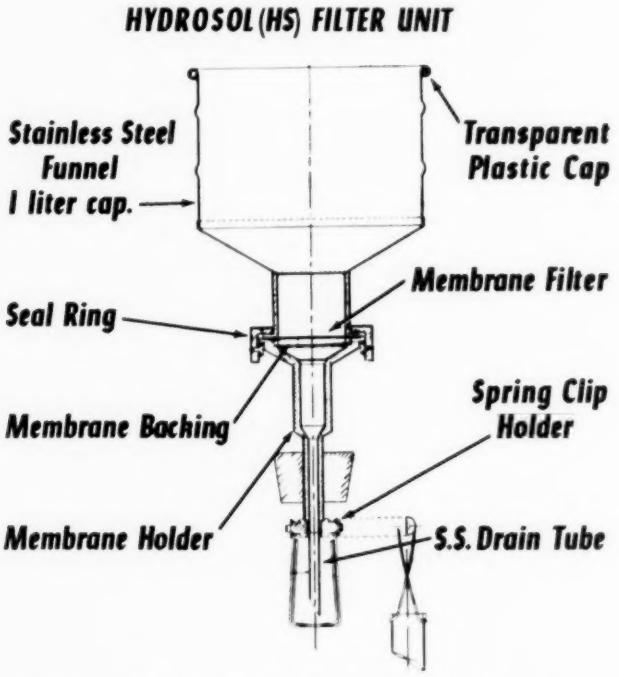

FIG. 1. Line drawing of Goetz hydrosol filtration apparatus.

between the supporting wire grid and the membrane in order to prevent rupture of the latter.

In air-sampling experiments, either a closed Seitz filter (for pressure and vacuum) or a Goetz-designed aerosol filter apparatus was used. The aerosol filter is similar to the hydrosol type, but the funnel of the latter is replaced by a closed top with air inlet, similar to that in the Seitz pressure-vacuum filter.

EXPERIMENTAL HYDROSOL FILTRATION

The filter membrane and several grades of Whatman filter paper of varying retentivity were tested with aqueous suspensions of *H. capsulatum* tuberculate spores for ability to retain these spores and for speed of filtration. In the retention tests, the filter disc was either cultured or examined microscopically, while the filtrate was cultured on both Sabouraud dextrose agar and Sabouraud dextrose broth. It was found that Whatman papers No. 3 and No. 50 and the membrane completely sterilized *H. capsulatum* spore suspensions, while Whatman No. 1 filtered out most of the spores but permitted some to pass through, as was evidenced by growth in the filtrate cultures. The maximum rate of filtration of tap water obtainable with the membrane filter apparatus, using an electric vacuum pump, was about 30 liters per hour. At a comparable pressure, Whatman No. 3 paper supported in a Seitz apparatus, either large or small, permitted a flow of 48 liters per hour. Application of greater pressures in an attempt to increase this rate resulted in tearing of the paper, although reinforcement of the No. 3 with 2 discs of No. 1 permitted a flow of 60 liters per hour without rupture. The filtration speed of the No. 50 paper in a Seitz apparatus approximated that of the No. 3, but the former sometimes permitted leakage around the periphery unless supplied with a rubber or cork gasket.

STAINING OF THE FILTER DISC

The staining procedure used for all filter discs was adapted from the Hotchkiss-McManus (periodic acid-Schiff) method for differentiation of fungus structures in animal tissue (**8**). The disc is removed with forceps from the filter apparatus and transferred to a small (60×15 mm.) clean Petri dish. The following reagents are in turn poured into the dish and discarded after the stated time intervals: periodic acid, 1% aqueous—5 minutes; tap water—3 changes totaling 5 minutes; Schiff reagent—5 minutes; potassium metabisulfite solution (5 ml. of 10% aqueous potassium metabisulfite and 5 ml. of normal hydrochloric acid in 100 ml. of distilled water; mixture to be prepared fresh daily)—3 changes of 5 minutes each; tap water—rinse twice, then apply fresh water for 5 minutes. The moist disc is then transferred to a 2×3 inch glass microscope

slide and covered with a 45 × 50 mm. coverslip, care being taken to exclude air bubbles. Water is added by capillarity if necessary. The preparation is examined systematically upon the compound microscope under low power (100 ×) with transmitted light. For critical examination of individual objects, high power (440 ×) may be used without significant loss of definition. During the examination, water is added as necessary to keep the area beneath the coverslip sufficiently moist for preservation of translucency, but care must be taken to avoid an excess of water which will cause the disc to float, thus interfering with sharp definition.

In such a preparation *Histoplasma* spores and other hyaline fungus structures are stained a deep pink or red and are strikingly visible against a very pale pink or colorless background (FIG. 2, a). Dematiaceous spores or hyphae, although not visibly stained, also stand out. Pollen grains take the stain well and details are particularly well defined. Examination of the membrane disc is facilitated by the presence of a ruled grid upon its surface. With the 16 mm. (10 ×) objective the diameter of the microscopic field is almost exactly half that of the grid units, with which most membranes come supplied, so that in surveying the disc under low power a guide line is always present at one edge of the field. When a suspected object is sighted it may be examined more critically with the 4 mm. objective.

Early in the course of this study it was suspected that, on account of the evenness of the membrane surface, some spores might be washed off during the staining process. That such was actually the case was confirmed by saving all reagents (except the Schiff stain) used in the processing, combining them, and later filtering the mixture through a No. 3 disc. Upon staining and examination of the latter, numerous spores were found. Some sort of pre-stain fixative was therefore necessary, which would not, however, interfere with the staining results. Haupt's adhesive (7) was found to suit the purpose. It is applied to the exposed membrane in a thin layer by means of a dropper and must be permitted to coagulate before initiation of the staining. Collodion (Collodion Flexible, U.S.P.) may also be used and has the advantage of rapid evaporation of the solvent, but it is difficult to apply evenly without disturbing the surface of the disc.

The Haupt fixative has been employed routinely also with the No. 50 paper, but is not considered necessary for the No. 3 paper, which has a coarse fibrous composition. As has already been pointed out, these filter papers, on account of their lesser homogeneity, are not so suitable for microscopic examination as the membrane, but may be employed satisfactorily and do permit excellent spore differentiation upon staining.

PERMANENT MOUNTING OF STAINED DISCS

Stained membranes and Whatman papers may be stored dry and subsequently rehydrated for examination, but repeated drying and moistening eventually impairs the sharp differentiation of spore details. For temporary storage a moist chamber may be used, but it is possible to make good permanent preparations by means of a mounting resin, as follows:

1. Allow the disc, with slide and coverglass, to air-dry.
2. Rim the coverglass with an excess of a xylene- or toluene-based mounting resin (HSR has been employed in this work).
3. Immediately apply xylene to the rim at one or more points until the xylene-resin solution has, by capillarity, saturated the entire disc.
4. Allow to harden, rimming with additional resin as needed.

Upon hardening of the mounting medium such a preparation remains transparent (FIG. 2, b), with spores readily discernible microscopically (FIG. 2, c–h). It is sometimes necessary to provide additional xylene or resin in order to cover denuded areas.

PRACTICAL WATER SAMPLING

Limited field trials have been undertaken with samples of drinking water, rain water, and river water from an area in Tennessee where there is a high prevalence of histoplasmin sensitivity among the human population. These samples were collected and filtered by Dr. L. D. Zeidberg of the Tennessee Department of Public Health, Nashville, Tennessee, and the filter discs were mailed to this laboratory for processing. As reported elsewhere (**4**), a tuberculate spore typical of those of *H. capsulatum* appeared on a membrane through which one liter of river water had been passed.

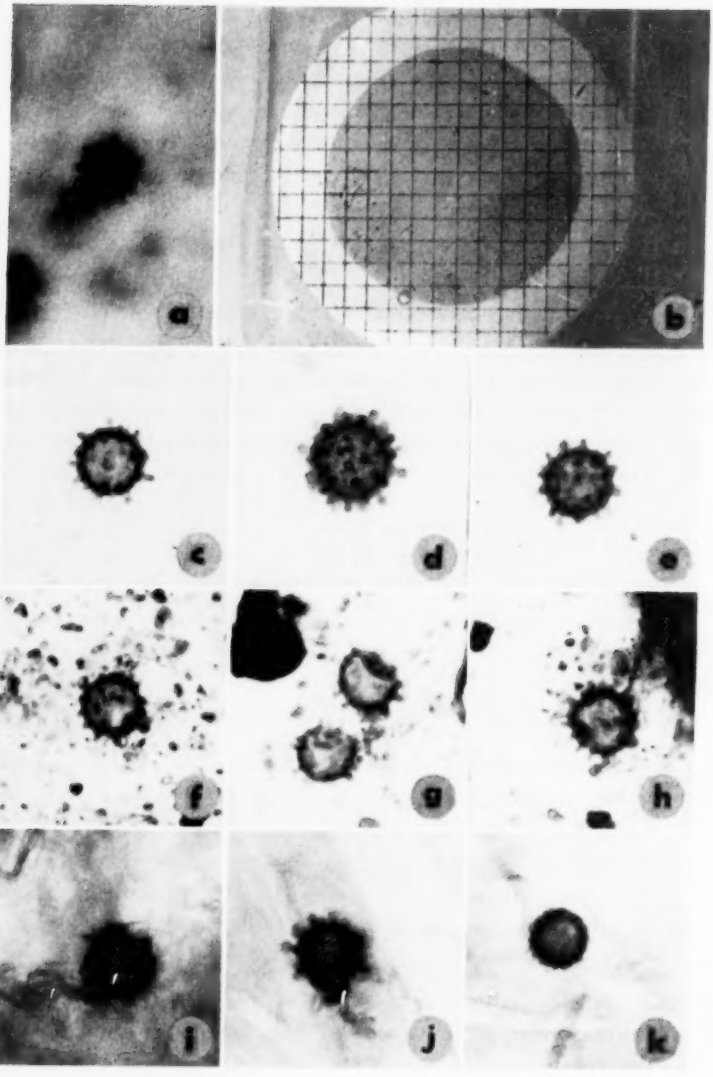

FIG. 2. All material stained by modified periodic acid-Schiff method. a. Tuberculate spore of *H. capsulatum* from soil, on membrane filter. Wet mount, × ca. 600. b. Permanent mount of stained membrane, × 1. c–e. Known *H. capsulatum* spores from artificial hydrosol, on membrane. Permanent mount, × 760. f–h. Spores from soil on membrane. Permanent mount, × 760. i–k. Known *H. capsulatum* spores from artificial aerosol, on Whatman No. 50 filter paper. Wet mount, × 760.

H. capsulatum has not been recovered from the other water sources, but these have yielded many other types of fungus spores, both hyaline and dematiaceous, including those of *Alternaria*, *Curvularia*, and other species, and large numbers of pollen grains. In practice, one-half to two liters of water have been filtered through each disc, but the usable volume is limited only by the degree of opacity produced by the sediment. In processing a series of samples where distilled water is not available for flushing the filter apparatus between specimens, the apparatus may be rinsed before each run with a small quantity of the material about to be filtered.

In order partially to answer the question of whether *H. capsulatum* spores remain viable in drinking waters for any considerable period of time, thereby constituting a possible health hazard, a series of viability experiments was conducted wherein various water samples, sterilized by Seitz-filtration, were seeded with known concentrations of these spores and sampled periodically by plating for *H. capsulatum* colonies. It was found that a small percentage of the spores remained viable for at least one month in distilled water and 3 weeks in tap water or untreated well water.

RECOVERY FROM SOIL OF *Histoplasma capsulatum* AND *Microsporum gypseum*

Four samples of soil (kindly made available by Dr. L. Ajello), aliquots of which had been found by the mouse-culture technique (1) to contain an infectious stage of *H. capsulatum*, were searched for the tuberculate spores of this fungus by the following procedure: a teaspoonful of soil was triturated in a sterile 25 × 150 mm. test tube with 30 ml. of a sterile 0.85% solution of sodium chloride. The suspension was permitted to settle for from two to several hours and then the entire clear supernatant was filtered through a membrane filter. This was fixed with Haupt's adhesive, stained, and examined. Typical well-stained tuberculate spores (FIG. 2, f–h), 9.5–12 μ in diameter (exclusive of the tubercules), were found in each of the soils, their number ranging from 1 or 2 on the entire disc, in the poorest sample, to an average of at least one per low-power field in the best. The spoonful of soil which had provided the highest count was then re-extracted with an additional 30 ml. of saline and was found again to yield a considerably reduced, but still

appreciable, number of spores. As a result of this and further repetitions, it has become evident that the flotation procedure employed is considerably less than 100 per cent efficient in extracting fungus spores from soil. Emmons (**3**) previously had recovered *H. capsulatum* spores from soil by means of a similar flotation technique, following which, however, portions of the supernatant were examined directly under the microscope. In the present studies such an examination also was successful with samples rich in spores, but the filtration method has proved to be more efficient. It is not known how many spores or other particles of *H. capsulatum* must be present in the soil inocula in order to infect mice, so that there has been no indication heretofore of the degree of infestation of soils with this organism. The membrane filter method would appear to offer an opportunity for quantitative determination, based on the number of spores present. A comparative evaluation of the two methods for detecting *H. capsulatum* in soil specimens is now under way in this laboratory.

An additional discovery of importance on one of the soil-filtration membranes was the presence of well-stained multicellular conidia typical of the dermatophyte, *Microsporum gypseum*. Resuspension of the soil aliquot which had yielded these spores, followed by streaking of a portion of the supernatant on selective media, resulted in the isolation of this fungus in pure culture. Details of these studies will appear elsewhere.

EXPERIMENTAL RECOVERY OF AIRBORNE SPORES OF *H. capsulatum*

A preliminary experiment on the filtration of artificial fungus aerosols has been concluded. A small quantity of dry *H. capsulatum* spores was mixed with a large volume of talcum powder in a 125 ml. side-arm suction flask equipped with a one-hole rubber stopper. The stopper was fitted with an L-shaped glass intake tube, cotton-plugged at its external orifice and narrowed to a small aperture at the opposite end, which was situated about 3 cm above the surface of the spore-talcum mixture. A length of glass tubing with rubber connecting-pieces led from the side-arm of this flask to the pressure inlet of a large (100 ml.) Seitz filter apparatus, containing a Whatman No. 50 paper disc and rubber gasket and inserted into

a one-liter suction flask. Gentle suction was applied to the side-arm of the large flask for several minutes, until a white coating of powder had begun to appear on the interior wall of the glass connecting-tube. Upon completion of the filtration the filter disc, visibly coated with talc, was moistened, transferred cautiously to a Petri dish, coated with Haupt's adhesive, and stained as above. Numerous well-stained scattered tuberculate spores (FIG. 2, i–k), resembling in all details those recovered from soil specimens and water, were strikingly visible against the unstained talc and filter paper.

FILTRATION OF *Coccidioides immitis* HYDROSOLS

Aqueous suspensions of arthrospores of *Coccidioides immitis* were filtered through membranes and through No. 50 paper fitted into a small Seitz apparatus. The filter discs were stained and the filtrates cultured. In this experiment, filtration through the membrane was more rapid than that through the Whatman No. 50 disc. Each served to retain most of the spores, but neither completely sterilized the suspension, as was shown by growth of *C. immitis* colonies in the filtrate cultures. Arthrospores upon both types of filter disc were well stained, but required tedious search on account of their minute size. Detection of the spores under low power was more difficult on the No. 50 filter, as a result of its lesser optical homogeneity and the trapping of some of the spores in the interstices of the fibers. However, under higher magnification (440 ×) the characteristic structures of *C. immitis* were readily found and easily recognized.

SUMMARY

The membrane filter affords a simple, rapid, and efficient method for detection of fungus spores in hydrosols and aerosols. The exposed membrane may be subjected to a selective stain and examined through the compound microscope by means of transmitted light. Details of this procedure are described.

Whatman filter papers of certain grades and Seitz filters may sometimes serve as acceptable substitutes for the more expensive and less generally available membranes and special membrane filter apparatus.

In field trials of this method, typical tuberculate spores of *Histoplasma capsulatum* have been recovered from several soil samples and from river water. Macroconidia of *Microsporum gypseum* have also been found in soil.

A method is described for preparing stained permanent transparent mounts of the filter discs.

Grateful acknowledgment is made to Mr. Lawrence B. Hall, Communicable Disease Center, Savannah, Ga., for having made available some of the equipment used in this study.

COMMUNICABLE DISEASE CENTER,
PUBLIC HEALTH SERVICE,
FEDERAL SECURITY AGENCY,
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EVIDENCE FOR SYNONYMY OF TORULA BERGERI AND PHIALOPHORA JEANSELMEI

A. Trejos

(with 2 figures)

During the course of the study we have made on 30 strains of etiologic agents of chromoblastomycosis, isolated by us in Costa Rica, we have observed, since the middle of 1950, cultures of *Torula bergeri* and pathological material from the case that was diagnosed in Canada by Berger and coworkers.[1] The comparative study of these cultures with those of other agents of chromoblastomycosis and maduromycosis has enabled us to make some observations summarized below.

We received from Dr. Carrión two cultures labeled "*Candida*-like sp. de Berger" with numbers *1074* and *1077*. Strain *1077* corresponds to the one isolated by Berger *et al.* (**1**) from the last biopsy performed on his patient in 1943. This strain has maintained the same macroscopical appearance that it had when first isolated; that is to say, dry, wrinkled and protruding colonies which do not penetrate the culture media, as described by Berger, Beadry and Gaumond (**1**) and Berger and Langeron (**2**). They readily separate in fragments, like powdered charcoal, when touched with the platinum loop (Fig. 1, a).

Strain *1074*, when received by us, showed the yeastlike moist appearance (Fig. 1, b) shown by the other strains isolated between 1936 and 1943. According to Berger *et al.* (**1**) and Berger and Langeron (**2**), this strain never developed aerial hyphae during the time they observed it, which was over eight years. However,

[1] The author wishes to express his appreciation to Dr. Arturo L. Carrión for his many kindnesses, including the cultures of *T. bergeri* that he sent to him, and to Dr. Carlton Auger of the Institute d'Anatomie Pathologique of the Université Laval of Quebec for the pathological material from the case of Berger and his coworkers.

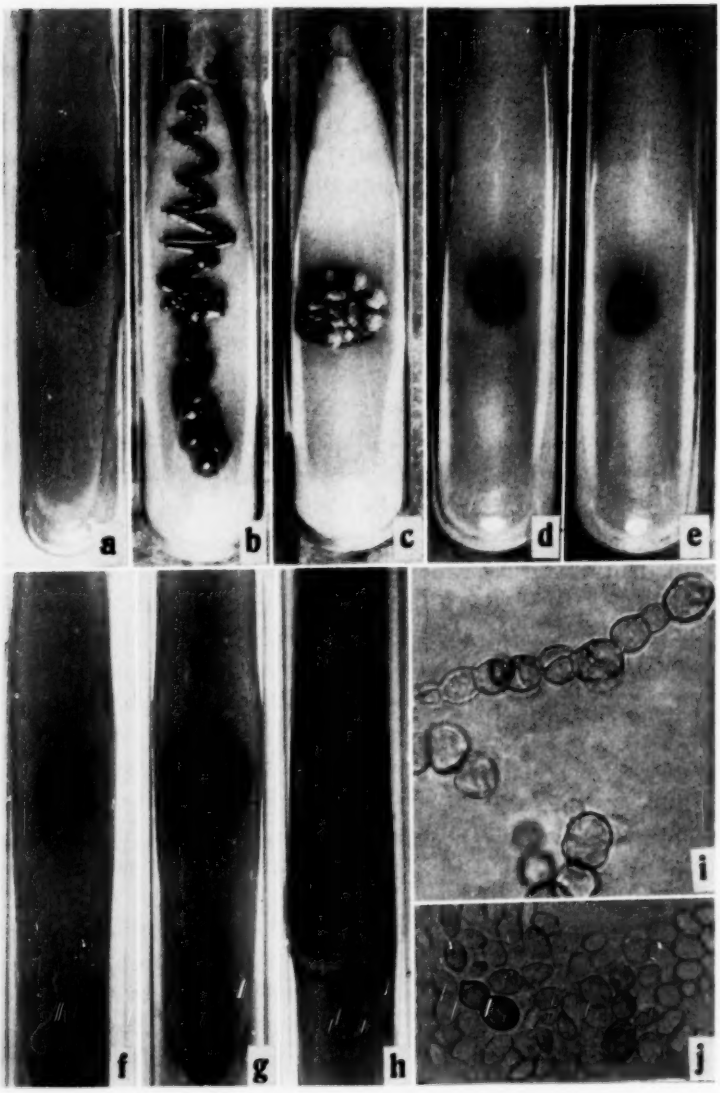

FIG. 1. a, *T. bergeri* No. *1077*. Culture two and a half months old on Sabouraud dextrose agar. b–h, *T. bergeri* No. *1074*. b, c. Cultures five weeks old on Sabouraud maltose agar. d. Culture five weeks old on corn-meal agar, taken from a sector with aerial hyphae. e. Culture five weeks

in one of the first transfers we made, we observed the formation of a sector which had short aerial hyphae. In subsequent transfers to Sabouraud dextrose and maltose agars from the sector with true aerial mycelium, the number of these sectors increased gradually (FIG. 1, c). When we made subcultures from the moist portion of the colony, the same appearance was maintained, while the cultures from the sectors with aerial hyphae became more and more moldy (FIG. 1, f, g, h). The growth in corn-meal agar was always slower than in the aforementioned media and the colonies were almost exclusively made up of submerged pseudomycelium, whether transferred from sectors with aerial hyphae (FIG. 1, d) or from moist and glabrous portions (FIG. 1, e).

The microscopic aspect of strain *1077* is at present the same as described by Berger *et al.* (**1**) and Berger and Langeron (**2**), as can be seen in the photomicrograph (FIG. 1, i). With strain *1074* this did not happen. Moist cultures on several media showed almost exclusively yeastlike cells, some with thicker and pigmented walls (FIG. 1, j), and slide cultures with corn-meal agar gave out pseudomycelium with blastospores similar to the ones observed in the genus *Candida* (FIG. 2, a) as described in papers of the authors mentioned above (**1, 2**).

The cultures with aerial hyphae showed microscopically a true, septate mycelium, from 1 to 3 μ in diameter with septa every 5 to 17 μ (FIG. 2, b). The slide cultures on Sabouraud maltose agar showed the *Pullularia* type of sporulation (FIG. 2, c) in which the conidia grew directly and pleurogenously on the hypha. It was also observed that, on the tips of the hyphae, spores were formed which remained in clusters. In this case there was no differentiation of the conidiophore, because there were no morphological changes with regard to the rest of the hypha (FIG. 2, d, e, g). Single or 2-celled conidiophores were formed perpendicular to or at an angle with the hyphae, usually immediately before a septum

old on corn-meal agar, taken from a yeastlike culture. f, g, h. Cultures two and a half months old on Sabouraud dextrose agar. i, *T. bergeri* No. *1077*. Culture four months old on Sabouraud dextrose agar; moniliform chains in which some of the cells show budding and others show septa; lactophenol, $\times$ 1000. j, *T. bergeri* No. *1074*. Culture four days old on Sabouraud dextrose agar; some cells show thicker and darker walls, lactophenol, $\times$ 1000.

(FIG. 2, f, h), which also showed clusters of conidia at their distal end. These conidiophores were cylindrical or bottle-shaped and they, as well as the hyphal ends which bore conidia, sometimes presented a very short extension or neck (FIG. 2, e, h, j) through which a small portion of protoplasm appeared to come out to form a conidium (FIG. 2, h, j). In some cases it was possible to observe a very slight cuplike broadening (FIG. 2, k). In others, the spores remained connected to the hyphae by very thin filaments which looked like cytoplasmic threads (FIG. 2, g).

Clusters of acrogenous conidia were found not only at the tips of cylindrical hyphae and conidiophores as previously described, but also at the end of moniliform chains of cells (FIG. 2, h, i) that were commonly seen in slide cultures and which were formed from yeastlike cells or spores of the inoculum. Also, some of the terminal cells of these chains presented a very short cylindrical neck (FIG. 2, l).

What we have described above clearly shows the evolution of a fungus from the characteristics of the so-called black yeasts to the filamentous stage with true aerial mycelium. This type of transformation has already been observed by several authors, as pointed out by Skinner, Emmons and Tsuchiya (**10**).

Berger and Langeron, in their paper on *Torula bergeri* (**2**), divide the so-called black yeasts into two groups: a) those which have a yeastlike stage at first and are later covered with aerial filaments, and b) those which they call true yeasts, without aerial filaments, and that give out a pseudomycelium which penetrates the medium. They place *Torula bergeri* among the latter group. We have already seen that *Torula bergeri* may produce a true aerial mycelium under certain special conditions which we have not as yet determined. Therefore, this separation, which already seemed somewhat artificial, loses its taxonomic value and we are inclined to agree with the opinion of Hansen and Lindner, who consider, according to Skinner *et al.* (**10**), ". . . that these black yeasts are but yeastlike growth forms of dematiaceous molds of the type of *Cladosporium*. . . ."

As years go by we see how pathogenic species which were originally classified as "black yeasts" are taken out of this group and given a more appropriate place among the filamentous demati-

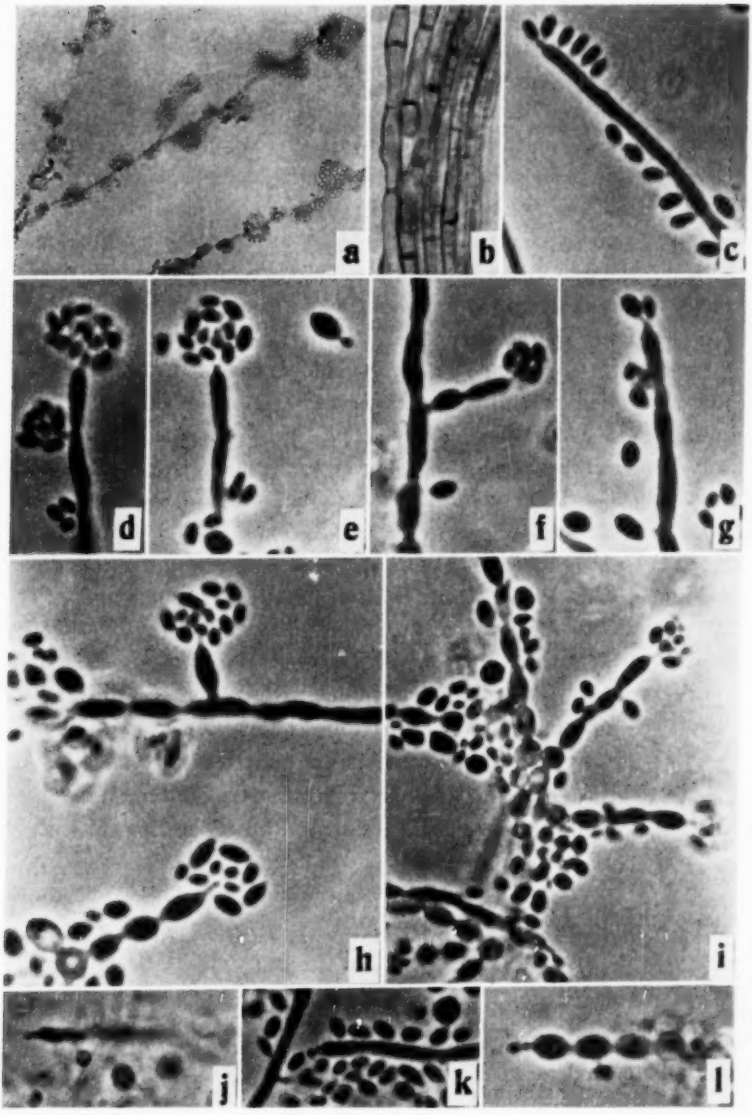

FIG. 2. *T. bergeri* No. 1074. a. Slide culture 8 days old on corn-meal agar, lactophenol, × 200. b. Culture five months old on Sabouraud glucose agar, lactophenol, × 1000. c-l. Several aspects of slide cultures on Sabouraud maltose agar, 11 days old, lactophenol, phase contrast, × 1200 except i, which is × 1000.

aceous fungi. This has happened with *Torula jeanselmei* Langeron and more recently with *Hormiscium dermatitidis* Kano, which was classified by Carrión (**3**) as *Fonsecaea dermatitidis* (Kano, 1937) Carrión, 1950. This is now also the case with *Torula bergeri*, to which we have to give a classification more in agreement with the characteristics previously described. Undoubtedly it has a remarkable morphological resemblance with *Phialophora jeanselmei* (Langeron) Emmons, according to the observations made by Emmons (**4**) and the more recent ones made by Mackinnon *et al.* (**7**), who have studied in detail the morphology and biology of *P. jeanselmei*. The biological characteristics of *T. bergeri*, as observed by Berger, Beaudry and Gaumond (**1**), Berger and Langeron (**2**) and by us, are in agreement with those observed by Emmons (**4**), Montemayor (**8**, **9**) and Mackinnon *et al.* (**7**) in *P. jeanselmei*.

We therefore consider *Torula bergeri* synonymous with *Phialophora jeanselmei* (= *Torula jeanselmei* Langeron, 1928 = *Pullularia jeanselmei* (Langeron) Dodge, 1935).

At present four strains of this species are known, the one studied by Langeron in 1928 (**6**) and the two observed by Emmons (**4**, **5**); all three isolated from mycetomas, and the fourth, called *T. bergeri*, isolated from a case of chromoblastomycosis (**1**, **2**).

We shall publish later on, together with a more detailed comparative study of the strains mentioned above, a discussion of the generic position of this polymorphous and interesting species. We shall also deal with the subject of atypical mycetomas and the atypical chromoblastomycosis of Canada caused by this fungus.

SUMMARY

The author studied *Torula bergeri* isolated by Berger and his coworkers in Canada, and felt that there were no specific differences between this fungus, which produced chromoblastomycosis, and *Phialophora jeanselmei*, one causative agent of mycetomas. Therefore he considers *Torula bergeri* as synonymous with *Phialophora jeanselmei*.

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STUDIES ON SOME SELENOPHOMA SPECIES ON GRAMINEAE [1]

JAI YOUNG PARK [2] AND RODERICK SPRAGUE [3]

(WITH 2 FIGURES)

Selenophoma species are common parasites of the grass family in the temperate and arctic parts of the world. Until recently this group had been little studied and only a few specimens had been collected by mycologists. Sprague and Johnson published information on the morphology of the genus on Gramineae (**8**, **9**, **10**, **11**, **12**). Allison confined his detailed investigations to *S. bromigena* (Sacc.) Sprague & A. G. Johnson (**1**, **2**, **3**). The present paper gives some of the more pertinent data of detailed comparative studies made not only on *S. bromigena* but on *S. donacis* (Pass.) Sprague & A. G. Johnson, *S. everhartii* (Sacc. and Sydow) Sprague & A. G. Johnson and *S. obtusa* Sprague & A. G. Johnson.

MATERIALS AND METHODS

Single-spore pure cultures of *Selenophoma* spp. were obtained from various members of the Gramineae collected in widely scattered parts of the Intermountain West. These cultures were maintained at 20° C. Those actually used in the investigations were limited to one to six isolations obtained from the following collections:

Selenophoma bromigena: on *Bromus inermis* Leyss from Pullman, Washington; *B. ciliatus* L. from Climax, Colorado; *B. carinatus* Hook. & Arn. from Pullman, Washington and Yellowstone Park, Wyoming.

[1] Scientific Paper No. 1071, Washington Agricultural Experiment Stations, Pullman. Project No. 449.

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S. donacis: on *Elymus glaucus* Buckl., Pullman, Washington; *Poa ampla* Merr., Pullman, Washington; *Triticum aestivum* L., Pullman, Rosalia and Colfax, Washington and Moscow, Idaho.

S. everhartii: on *Calamagrostis rubescens* Buckl., Angle Peak, Washington; *Festuca myuros* L., Skyline Drive, Idaho.

S. obtusa: on *Agropyron spicatum* (Pursh) Scribn. & Sm., Angle Peak, Washington; *Elymus glaucus* Buckl., Cedar Pass, California.

Spore germination studies were made of all of these isolates, using 100 spores from each collection. Isolation onto potato-dextrose agar was done by means of a dry glass micropipette. Studies were conducted at temperatures of 1, 6, 10, 15, 20, 25 and 30° C. Spore germination and mycelium growth rate were compared at several different pH levels, ranging from 3.0 to 8.0. The pH of the media was adjusted with HCl and NaOH, using a Beckman Electric pH meter for recording.

Nuclear division in the germinating spores of *S. donacis* from wheat collected at Pullman, Washington, was studied at 20° C. Transfer was made in the same way as in the spore germination studies. The spores that were examined were stained with Heidenhain's iron haematoxylin from 5 to 25 hours after isolation.

Relative humidity studies were made by growing the 100 samples of dry spores on a glass slide in air bubbled through various solutions to maintain the following approximate percentages of relative humidity: 100 % (water only), 95% (sodium sulfite), 90% (zinc sulfate), 81% cent (ammonium sulfate) and 76% (sodium chlorate) relative humidity (**5**, **6**, **7**).

Cross inoculation studies were made in the greenhouse and out-of-doors by spraying seedlings, which were 20 to 30 days old, with pycnospore suspensions obtained from the various pure cultures listed above. The most satisfactory spray chamber was a wooden box with dimensions of $3 \times 2 \times 2.5$ feet, embedded in the ground and covered with a muslin cloth. Humidity was maintained by keeping a six-inch floor layer of peat moss dampened with water. At a favorable temperature of 65–70° F. the incubation period was about 72 hours. No artificial light was used. Allison (**3**), among others, found that spores of *S. bromigena* germinated readily in the dark.

Forty-three species of Gramineae in fifteen genera were used in cross-inoculation trials. These are shown in Tables II and III.

The data obtained in these studies are given in detail in an unpublished manuscript by the senior writer [4] filed at Washington State College. The present paper briefly summarizes most of these data plus some additional information.

EFFECT OF TEMPERATURE

Spores of most of the species germinated in the temperature range of 15–25° C. within 20 to 30 hours after isolation. Within 40 hours from 60 to 100 per cent of the spores had germinated. At 30° C. all of the fungi were inhibited, only 12 to 20 per cent of the spores germinating after 40 hours. Most of the fungi had a minimum temperature for spore germination of 5–6° C. However, some spores of *S. bromigena* from *Bromus inermis* and *S. donacis* from *Poa ampla* did not germinate at temperatures below 10° C. after 40 hours incubation.

The largest number of spores of *S. everhartii* from *Calamagrostis rubescens* and *Festuca myuros* germinated at 25° C. while the isolates of *S. donacis*, *S. bromigena* and *S. obtusa* tended to have their maximum germination at 18–22° C. Allison (3) reported that *S. bromigena* had an optimum temperature of 19–22° C. for spore germination with the minimum for spore germination between 3° C. and 5° C.

The relationship between hyphal production and secondary conidial production was compared at temperatures of 1, 6, 10, 15, 20, 25 and 30° C. (Table I). In general, the production of secondary conidia increases with an increase in temperature. At 20° C. or higher *S. donacis* from wheat and *Poa ampla* and *S. bromigena* from *Bromus carinatus* produced masses of secondary conidia (pseudopionnotes) from germ tubes without production of hyphae. The mass of conidia were formed at the septate portion of the germ tube (FIG. 1, D).

[4] Jai Young Park. Comparative Studies of Selenophoma Leaf Spot Diseases on Certain Grasses. Thesis submitted to Washington State College as partial fulfillment of the requirements for the degree of Doctor of Philosophy. The author expresses his deep appreciation of the aid of George W. Fischer, Geneva Fischer, and C. Gardner Shaw in the preparation of this thesis.

TABLE I

THE RELATIVE AMOUNT OF HYPHAE (−) AND CONIDIA (+) PRODUCED UNDER DIFFERENTIAL TEMPERATURE RANGE ON POTATO DEXTROSE AGAR AT 20° C. AFTER 70 HOURS' INCUBATION

Isolate/Host	Temperature range (°C.)						
	1	6	10	15	20	25	30
Selenophoma bromigena[5] *Bromus inermis*		− − +	− − + +	− − + +	− + + +	− − − −	− − − −
Selenophoma bromigena *Bromus carinatus*	−	− −	− − + +	− + + +	+ + + +	+ + + +	+ + + +
Selenophoma donacis *Triticum aestivum*		− −	+ +	+ + +	+ + +	+ + + +	+ + + +
Selenophoma donacis *Poa ampla*	−	− −	− + +	− + + +	+ + + +	+ + + +	+ + + +
Selenophoma everhartii *Festuca myuros*		− −	− − − +	− − − +	+ + − −	− − − −	− − − −
Selenophoma everhartii *Calamagrostis rubescens*		− −	− − +	− − − +	+ + − −	− − − −	− − − −
Selenophoma obtusa *Agropyron spicatum*		− −	− − − +	− − − +	+ + − −	− − + +	− − + +

[5] Relative amount of hyphae (−) or conidia (+) shown by number of dashes (−) or plus marks (+)

In general, relative development of hyphal production increased at lower temperatures as, inversely, conidial production fell off. Some cultures of *S. bromigena* from *Bromus inermis* from Pullman produced somewhat aberrant conidia at temperatures as low as 6° C.

COMPARATIVE MORPHOLOGY OF THE GERMINATING SPORE

There was considerable variation in the process of spore germination in the several isolations. Similar results are reported in the literature. Darley (**4**) reported that septation occurs in the pycnospore before germination on nutrient media but not in distilled water. In our trials pycnospores of *S. bromigena* from *Bromus inermis* on potato-dextrose agar produced germ tubes sometimes after one septum was formed in the spore, or in some cases before any septation had formed. These germ tubes developed from any portion of the pycnospore about 10 hours after incubation at 20° C. Allison (**1**) reported that germ tubes formed on either end of the spores after 12 hours.

Sometimes in *S. bromigena* from *B. inermis* one or two oval nodes are formed at the terminal portion of the germ tube after 20

hours (FIG. 1, A). Sometimes digitately branched hyphae occur after 25 hours (FIG. 1, B). These hyphae increase in number, forming a cluster which is a pycnidial initial.

There were some differences noted among some of the isolates. For instance, *S. bromigena* from *Bromus carinatus* developed three or four septa in the spore at the start of germination (FIG. 1, C). The hyphae from these germinating spores formed numerous cross walls. In the case of *S. everhartii* from both *Calamagrostis* and *Festuca* the spores germinated without previous septation.

In *S. obtusa* from *Agropyron spicatum* the spores became conspicuously swollen within an hour. Germ tubes were formed from the middle portion of the spores or from both ends after about 12 hours incubation at 20° C.

EFFECT OF ATMOSPHERIC HUMIDITY ON SPORE GERMINATION

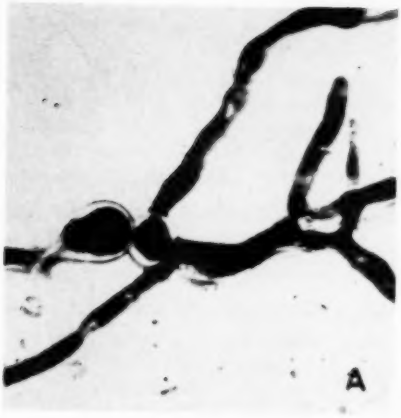
The spores of all isolations grew well at 100% relative humidity and appreciably less at lower humidities. At 81% humidity the growth was very slow. Allison (3) suggested that a film of water on the leaves at the time of infection and continued high humidity was necessary for rapid development of *S. bromigena* on *Bromus inermis*. This is indicated by our results.

EFFECT OF pH ON SPORE GERMINATION AND COLONY GROWTH

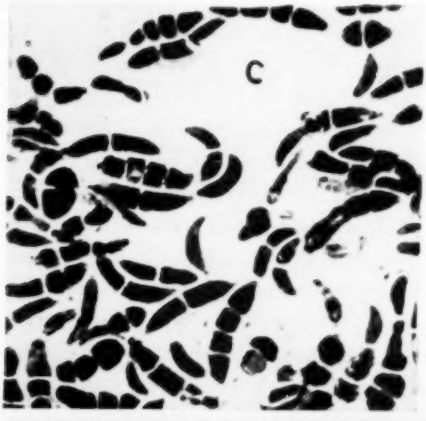
With few exceptions, spores of all species of *Selenophoma* germinated well at p ranges of 4.5–8.0, with the optimum at 5.0–6.0. At pH 3.0 spore germination decreased sharply.

Colonies of all species grew well at pH 4.0–8.0 with the optimum for all except *S. bromigena* at pH 5.5. *S. bromigena* from all hosts had an optimum range of pH 4.5–5.0.

FIG. 1. Germinating spores and pure cultures of *Selenophoma*. A. Spore germination of *S. bromigena* from *Bromus inermis*, 12 hours after isolation, ×700. B. *S. bromigena* from *B. inermis* showing digitate branching of hyphae, 25 hours after isolation, ×750. C. Germinating spores of *S. bromigena* from *Bromus carinatus* showing multiple septation 15 hours after isolation, ×750. D. Showing secondary conidial formation in *S. donacis* from *Agropyron cristatum*, 24 hours after isolation, ×750. E. Pure culture of *S. bromigena* on potato-dextrose agar, Race 1. F. Pure culture of *S. bromigena*, Race 2.


A


B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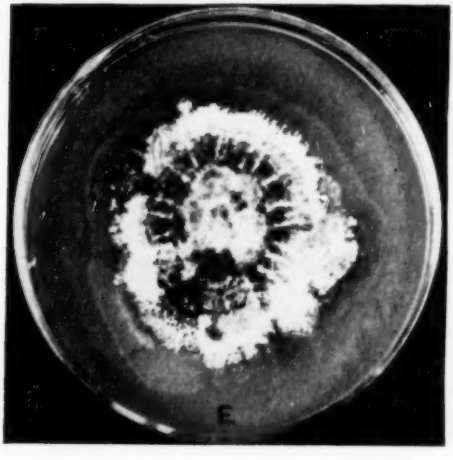
D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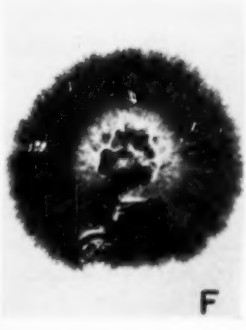
F

ADDITIONAL INFORMATION ON MACROSCOPIC APPEARANCE OF THE COLONIES

Sprague and Johnson (**12**) and Allison (**3**) have detailed information on the general appearance of isolations of most of the fungi handled in the current paper. Such data will not be repeated here.

It was found that a relatively high humidity could be maintained by sealing the petri dish cultures with rubber band seals. All of the fungi studied were slow growing, requiring two months to reach the margin of a 100 mm. petri dish at 20° C.

S. bromigena isolated from *Bromus carinatus* had a somewhat more carbonaceous appearance than those from *B. inermis* (compare FIGS. 1, E and 1, F). Isolates of *S. bromigena* from *B. ciliatus* from Climax, Colorado resembled those from *B. inermis* from the Palouse region and from Minnesota (**3**).

Isolates of *S. everhartii* from *Festuca myuros* produced flat, flesh-tinted, subcottony colonies with several rings of purple-brown to creosote-brown color at the margins and with strongly tinted vinaceous substrata. The small, black spore masses formed in the central area of the colony. Some colonies isolated from the same material had a flat, smooth surface of burnt-orange with no subcottony overgrowth. The first-mentioned type of colony resembles isolates of *S. everhartii* obtained from *Trisetum spicatum* (L.) Richt. by Sprague and Johnson (**12**) while the burnt-orange kind is similar to colonies of *S. everhartii* obtained from *Deschampsia danthonioides* (Trin.) Munro (**12**). The isolates obtained from *Calamagrostis rubescens* resembled these also except that the burnt-orange colonies from *Calamagrostis* developed a thin overgrowth of white mycelium.

GROWTH RATES OF THE SELENOPHOMA CULTURES ON POTATO-DEXTROSE AGAR

Experience with this group has shown that unless all isolates have been subjected to the same period of time in culture since their original wild state the comparative rate of growth of colonies of the same species may vary appreciably. While the studies herein briefly reported have avoided this to a considerable extent, we are still unsatisfied that all the differences noted are significant. However, in

general, *S. obtusa* grew almost twice as fast as isolates of *S. everhartii*. In a period of seven weeks the isolates of *S. obtusa* from *Agropyron spicatum* averaged 75 mm. in diameter while those of *S. everhartii* from *Festuca* and *Calamagrostis* averaged 33–35 mm. Cultures of *S. bromigena* and *S. donacis* grew at an intermediate rate of 47–56 mm. diameter in the same period of time.

PATHOLOGICAL HISTOLOGY

This work was limited to *S. bromigena* invading inoculated leaves of *Bromus inermis* and *B. carinatus*. The results confirmed, in the main, the detailed study by Allison (**3**). In the present studies the host penetration was through the stomatal openings. Sometimes masses of hyphae accumulate on the surface of the closed stomatal opening, invading the host after the stoma opened.

NUCLEAR BEHAVIOR DURING SPORE GERMINATION

The spores of *Selenophoma donacis* from *Triticum aestivum* were isolated on potato-dextrose agar at 20° C. and the nuclear division in the germinating spore was observed during the period of germination.

The spores were removed and stained with Heidenhain's haematoxylin, at 5–10, 10–15, 15–20 and 20–25 hours after they had been isolated on potato-dextrose agar. Nuclear division took place 5–10 hours after isolation (FIG. 2, A, B) and a septum developed between the two nuclei in the spore. One of these nuclei re-divided and at the same time a germ tube formed from the terminal portion of the spore 10–15 hours after isolation. One of the newly divided nuclei moved into the germ tube but the other nucleus remained in the spore body (FIG. 2, C). The nucleus in each half of the spore divided at the same time or about 15–20 hours after isolation (FIG. 2, D), and a germ tube resulted from each end of the spore. Further development of nuclei is shown in FIG. 2, E.

RESULTS OF INOCULATIONS

The results of cross-inoculation trials are partly shown in TABLES II and III. In addition to the inoculation results given in TABLES

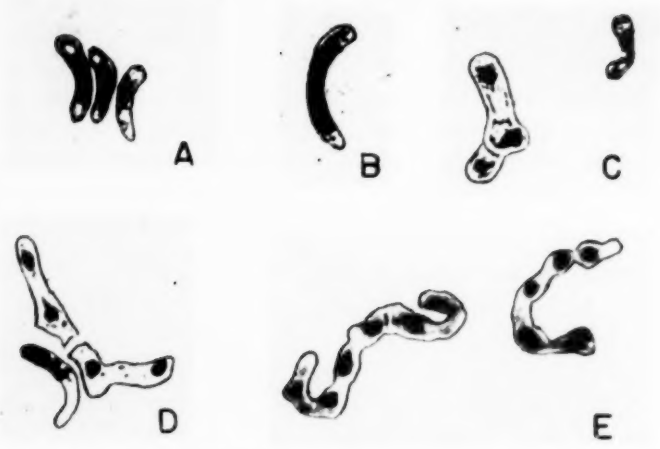

FIG. 2. The nuclear behavior during spore germination of *Selenophoma donacis* from *Triticum aestivum*. A. About 5–10 hours after isolation. B. Ten hours after isolation. C. 10–15 hours after isolation. D. 15–20 hours after isolation. E. 20–25 hours after isolation.

II and III, the following species were all inoculated with spore suspensions of the fungi listed in TABLES II and III and with entirely negative results: *Agropyron inerme* (623 leaves), *A. repens* (697 leaves), *A. sibiricum* no. *27* (1006 leaves), *Arrhenatherum elatius* no. *492* (804 leaves), *Bromus vulgaris* no. *2640* (966 leaves), *Festuca pacifica* (941 leaves), *Phalaris arundinacea* (688 leaves), *Phleum pratense* (776 leaves), *Poa compressa* (977 leaves), *Sitanion hystrix* no. *5505* (577 leaves), *Stipa columbiana* no. *1703* (761 leaves), *Stipa comata* no. *4903* (662 leaves) and *Stipa viridula* no. *3085* (518 leaves).

Most of the results which are listed above and in TABLES II and III are self-evident. The great majority of the isolates were restricted to their original hosts or to closely related ones. There were a few exceptions. *S. donacis* from *Triticum aestivum* attacked *Poa pratensis* but *S. donacis* from *Poa ampla* did not attack *Triticum*. Sprague (8) previously reported flecking on *Poa pratensis* from *S. donacis* from wheat from the Palouse region. This suggests one possible source of the occasional outbreak of *Selenophoma* on wheat in the Palouse region of Washington and Idaho. The iso-

late of *S. donacis* from *Elymus* is apparently a distinct race from that on wheat and *Poa* because it was confined to *Elymus* with only flecking on wheat and it was unable to attack *Poa* spp. The race on wheat in the Palouse region is called Race 1, that on *Poa ampla* at Pullman Race 2, and that on *Elymus* at Pullman is Race 3. The fact that Race 1 is able to attack *Poa pratensis* indicates affinity to Race 2, but Race 2 did not attack *Poa pratensis* in the current trials. All of these races appear at least as clear-cut as the considerably more minutely segregated ones of stem rust (*Puccinia graminis* Pers.).

Isolates of *S. bromigena* from *B. inermis*, *B. carinatus* and *B. ciliatus* were confined to brome grasses in our studies. The isolates from *Bromus carinatus* did not parasitize most collections of *B. inermis* except nos. *5792* and *8024* which were mildly attacked. The "carinatus" strain appears to be a relatively weak race of *S. bromigena*. It attacked *B. carinatus* in only 26 out of 148 leaves. The race on *B. inermis* would not attack true *B. carinatus* but it did attack one strain each of *B. marginatus* and *B. polyanthus* which are almost identical with *B. carinatus*. Strangely enough, the weak race on *B. carinatus* caused injury on *B. brizaeformis* and on *B. tectorum* while the race from *B. inermis* did not attack it. The common race in the Palouse on *B. inermis* is called Race 1 of our series and the uncommon race on *B. carinatus* is Race 2. Perhaps it only deserves a subclass, such as Race 1-B; but on the strength of our present available data, it seems simpler to classify it as a distinct race. *Selenophoma* on *B. carinatus* is uncommon in the West but seems to be increasing slowly. Allison (**3**) did not obtain infection on this host with material from *B. inermis*, indicating that the material of his more parasitic strain was comparable to our Race 1. The isolate from Colorado material of *B. ciliatus* possibly should be left unclassified because no available seed of *B. ciliatus* would germinate. On the other hosts it only weakly attacked the *5792* strain of *B. inermis*. Since it did not attack *B. marginatus* no. *10,708* as did Race 2, it would appear that it was another weak Race and can be called Race 3.

Selenophoma obtusa isolated from representatives of the two closely related genera *Agropyron* and *Elymus* were confined to

TABLE II

RESULTS OF CROSS-INOCULATION EXPERIMENTS WITH SELENOPHOMA SPP. ON VARIOUS GRASSES

Plant inoculated	Number of leaves infected (+) or not infected(−) by:											
	Selenophoma bromigena						*Selenophoma donacis*					
	Isolated from *Bromus inermis* (7)[2]		Isolated from *Bromus carinatus* (2)		Isolated from *Bromus ciliatus* (1)		Isolated from *Poa ampla* (3)		Isolated from *Elymus glaucus* (4)		Isolated from *Triticum aestivum* (5)	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
Agropyron cristatum	0	54	0	38	0	32	–	–	–	–	–	–
Agropyron spicatum 2719, 737, 6409[1]	0	141	0	141	0	104	0	154	0	123	0	88
Agropyron trachycaulum	0	79	0	56	0	77	0	56	0	34	0	65
Bromus anomalus 8027	0	136	0	181	0	76	0	34	–	–	–	–
Bromus brizaeformis	0	256	34	20	0	67	0	86	0	25	0	38
Bromus carinatus	0	232	26	122	0	89	0	35	0	47	0	49
Bromus erectus 4684	0	207	18	154	0	38	–	–	–	–	–	–
Bromus erectus 2336	19	169	0	193	0	32	–	–	–	–	–	–
Bromus inermis 3053	0	321	0	213	0	82	0	36	0	42	0	25
Bromus inermis 3197	74	169	0	101	0	65	0	42	0	36	0	45
Bromus inermis 5792	104	74	20	116	8	98	0	24	0	37	0	49
Bromus japonicus 1733	0	187	0	131	0	131	0	89	0	34	0	43
Bromus marginatus 8040	27	141	0	243	0	74	0	38	0	64	0	49
Bromus marginatus 5355	6	178	0	121	0	89	0	21	0	87	0	96
Bromus marginatus 2133	0	210	0	262	0	66	0	32	0	67	0	81
Bromus marginatus 3972	44	274	0	281	0	59	0	65	0	87	0	36
Bromus marginatus 7253	26	58	0	134	0	67	0	37	0	98	0	62
Bromus marginatus 10,708	0	282	28	144	0	97	0	34	0	88	0	48
Bromus marginatus 11,206	0	198	0	201	0	145	0	29	0	65	0	77
Bromus marginatus 8024	30	123	22	74	0	83	0	63	0	55	0	98
Bromus mollis 1728	0	227	0	242	0	87	–	–	–	–	–	–

TABLE II—*Continued*

Plant inoculated	Number of leaves infected (+) or not infected (−) by:											
	Selenophoma bromigena						*Selenophoma donacis*					
	Isolated from *Bromus inermis* (7)[2]		Isolated from *Bromus carinatus* (2)		Isolated from *Bromus ciliatus* (1)		Isolated from *Poa ampla* (3)		Isolated from *Elymus glaucus* (4)		Isolated from *Triticum aestivum* (5)	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
Bromus polyanthus 7477	17	93	0	221	0	98	0	36	0	67	0	34
Bromus tectorum	0	287	8	14	0	45	0	21	0	35	–	–
Bromus tomentellus 2447	0	285	0	191	0	52	0	21	0	21	0	31
Calamagrostis epigeios (L.) Roth.	0	22	0	47	0	43	0	32	0	39	0	54
Dactylis glomerata 8775	0	213	0	245	0	88	0	59	0	121	0	110
Elymus canadensis 3889	0	81	0	89	0	39	0	121	6	50	0	89
Elymus glaucus 2662	0	92	0	49	0	19	0	187	28	12	0	112
Festuca elatior 3161	0	123	0	201	0	36	0	43	0	98	0	38
Festuca ovina	0	98	0	56	0	39	0	59	0	49	0	56
Festuca pacifica	0	145	0	167	0	22	0	151	0	65	0	33
Hordeum vulgare	0	111	0	121	0	39	–	–	–	–	–	–
Hordeum vulgare	0	39	0	36	0	11	0	39	0	39	0	42
Hordeum vulgare	0	221	0	131	0	49	–	–	–	–	–	–
Hordeum vulgare	0	225	0	312	0	39	0	41	0	47	0	33
Poa ampla	0	138	0	203	0	34	21	40	0	87	0	73
Poa pratensis 3128	0	65	0	34	0	32	0	136	0	81	40	64
Secale cereale	0	113	0	289	0	89	0	76	0	58	0	39
Secale cereale	0	210	0	149	0	33	–	–	–	–	–	–
Stipa thurberiana 3710	0	18	0	56	0	14	0	36	0	34	0	59
Triticum aestivum	0	213	0	311	0	49	0	55	0[3]	69	44	40

[1] These numbers are the accession numbers of the Pullman (Wash.) Nursery Unit of the Soil Conservation Service, United States Department of Agriculture, whose aid in furnishing seed for these trials was invaluable.

[2] The figures in parentheses refer to number of pure culture isolations that were used in the trials.

[3] Flecking only.

TABLE III

RESULTS OF CROSS-INOCULATION EXPERIMENTS WITH SELENOPHOMA SPP. ON VARIOUS GRASSES

Plant inoculated	Number of leaves infected (+) or not infected (−) by:							
	Selenophoma obtusa				*Selenophoma everhartii*			
	Isolated from *Agropyron spicatum* (1)		Isolated from *Elymus glaucus* (1)		Isolated from *Festuca myuros* (1)		Isolated from *Calamagrostis rubescens* (1)	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
Agropyron cristatum	0	115	0	87	0	56	0	76
Agropyron spicatum 2719	36	35	0	124	0	89	0	45
Agropyron spicatum 737	0	145	0	113	0	53	0	79
Agropyron spicatum 6409	0	89	0	48	0	49	0	39
Agropyron trachycaulum	0	115	54	112	0	66	–	–
Bromus anomalus 8027	–	–	–	–	–	–	–	–
Bromus brizaeformis	0	26	0	47	0	78	–	–
Bromus carinatus	0	87	0	38	0	37	0	34
Bromus erectus 4684	0	34	0	81	0	59	0	46
Bromus erectus 2336	–	–	–	–	–	–	0	34
Bromus inermis 3053	0	19	–	–	0	26	–	–
Bromus inermis 3197	0	56	–	–	0	45	–	–
Bromus inermis	0	118	0	201	0	46	–	–
Bromus japonicus 1733	–	–	–	–	–	–	0	56
Bromus marginatus 8040	0	47	–	–	0	78	–	–
Bromus marginatus 2133	–	–	–	–	0	45	0	89
Bromus marginatus 3972	0	49	–	–	0	86	–	–
Bromus marginatus 7253	0	87	0	69	0	82	0	69
Bromus marginatus 10,708	0	32	0	45	0	96	0	56
Bromus marginatus 11,206	0	53	0	64	0	62	0	63
Bromus marginatus 8024	0	53	0	59	–	–	–	–
Bromus mollis 728	0	24	0	39	–	–	–	–
Bromus polyanthus 7477	0	39	0	89	0	47	0	88
Bromus tectorum	0	56	0	26	–	–	–	–
Bromus tomentellus 2447	0	29	–	–	–	–	–	–
Calamagrostis epigeios	0	55	0	23	0	118	8	28
Dactylis glomerata	0	92	0	54	0	79	0	24
Elymus canadensis	10	38	0	54	0	54	0	82
Elymus glaucus	0	43	45	236	0	27	0	42
Festuca elatior	0	34	0	37	0	42	0	90
Festuca ovina	0	44	0	41	10	42	0	69
Hordeum vulgare	0	65	0	67	–	–	–	–
Hordeum vulgare	0	45	0	39	–	–	–	–
Hordeum vulgare	0	23	0	43	–	–	–	–
Hordeum vulgare	–	–	0	13	–	–	–	–
Poa ampla	0	123	0	76	0	88	0	42
Poa pratensis	0	97	0	92	0	18	0	87
Secale cereale	0	67	0	54	–	–	–	–
Secale cereale	0	78	0	34	–	–	–	–
Stipa thurberiana	0	23	–	–	0	43	0	23
Triticum aestivum	0	65	0	98	–	–	–	–

these genera in their host range (TABLE III). The isolate from *Agropyron* could attack its original host and also *E. canadensis*. The isolate from *Elymus* could attack *A. trachycaulum* and its original host. The results do not indicate any racial difference in these two isolations; they may be considered as one strain.

The somewhat scanty trials with *S. everhartii* indicated that the isolate from *Festuca myuros* was confined to fescue and the isolate from *Calamagrostis* to that genus.

SUMMARY

The spores of most species of *Selenophoma* have an optimum temperature range for germination of 15–25° C. and a minimum of 6° C. except *Selenophoma bromigena* on *Bromus inermis* which germinated appreciably at 1° C. *Selenophoma everhartii* is the species best adapted to high temperatures of any of the graminicolous *Selenophoma* that were studied. Its optimum temperature for spore germination was 25° C. Its spores germinated slowly, requiring 40 hours' incubation to reach maximum spore germination.

Most species and varieties of *Selenophoma* produced some secondary conidia as well as hyphae at temperatures of 15–20° C. However, the following isolates characteristically produced only hyphae, without conidia, at 25° C. or higher temperatures: *Selenophoma bromigena* from *Bromus carinatus* and *S. everhartii* from *Festuca myuros*, and *Calamagrostis rubescens*.

In different stages of development starting with pycnospore germination, *Selenophoma bromigena* from *Bromus inermis* produced one or two oval nodes at the terminal portion of the germ tube (20 hours after isolation), the characteristic palm-shaped branching hyphae (25 hours later), and then hyphal cluster formation. This species also produced the most abundant hyphae of any of the species or varieties of *Selenophoma*. *S. bromigena* from *B. carinatus* was distinguished by the formation of several septations in the spore before germination.

In general, all species and varieties of *Selenophoma* germinated most rapidly at 100% relative humidity, but slower germination occurred even down to 81% humidity.

Most of the species and varieties of *Selenophoma* had an optimum pH of 5.0–6.0. However, *Selenophoma bromigena* had optimum ph ranges of 4.5–5.0.

S. obtusa on *Agropyron spicatum* grew more rapidly and vigorously than any of the isolates, growing approximately two times faster in diameter of the colony than *S. everhartii* which is the slowest growing fungus in the genus *Selenophoma* on Gramineae.

Selenophoma bromigena from *Bromus inermis* and *B. carinatus* penetrated through the stomatal chamber in all cases by forming appressoria. The mycelium grew intercellularly. The hyphae in the subcuticular region formed secondary pycnidia. Pycnidia appeared two weeks to 20 days after inoculation.

In *Selenophoma donacis* on *Triticum aestivum* nuclear division took place 5 to 10 hours after isolation on potato-dextrose agar, and a septum developed between the two nuclei of the spore. One of these nuclei redivided and at the same time a germ tube formed. The germ tube developed from the terminal portion of the spore, 10 to 15 hours after isolation.

The optimum temperature range for the infection of hosts by spore suspensions was 65–70° F., with an incubation period of 72 hours. The host plant was most susceptible when the 20–30-day-old plant was 3–4 inches tall. A subterranean inoculation chamber was the most satisfactory equipment used in cross inoculation studies.

Selenophoma bromigena did not attack any hosts other than members of the genus *Bromus*. It had three races, number one on *Bromus inermis*, a weakly pathogenic Race 2 on *Bromus carinatus* and another weak one, Race 3, on *B. ciliatus*.

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MICROMYCES AND SYNCHYTRIUM

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The genus *Micromyces* was established by Dangeard in 1888 for a minute chytrid parasite which he found in the alga *Zygogonium* and named *M. zygogonii*. This and other similar parasites of the Conjugatae were observed earlier by Thwaites (**1846**), Shadbolt (**1852**), Smith (**1853**), Pringsheim (**1857**), deBary (**1858**) and Reinsch (**1875**, **1879**), but these workers were not certain of the parasitic nature of these organisms and described the spiny prosori and resting spores as asteridia and astrospheres. Dangeard recognized the close similarity of *Micromyces* to the older and better known genus *Synchytrium* (deBary and Woronin, **1863**), but because of the former's minute size, aquatic habitat and its formation of a sporangial sorus outside of the initial cell he regarded it as a distinct and valid genus. *Micromyces zygogonii* was subsequently reported by Dangeard (**1891**), de Wildeman (**1891**), Petersen (**1909**, **1910**), Minden (**1911**), Denis (**1927**), Huber-Pestalozzi (**1931**), Heidt (**1937**), Couch (**1931**, **1937**), Sparrow (**1943**), Canter (**1949**) and Rieth (**1950A**), and five new species and one variety were added to the genus by Scherffel (**1904**), Skvortzow (**1925**), Couch (**1937**), Sparrow (**1943**), Canter (**1949**), and Rieth (**1950B**).

Most of these workers recognized the fundamental similarity of *Micromyces* to *Synchytrium*, and as early as 1892 Fischer noted that *Micromyces* should be placed in his new family Merolpidiaceae (Synchytriaceae) if Dangeard's (**1888**) observations were confirmed. He further stated (p. 72): "Er wurde der Sectio *Pycnochytrium* der Gattung *Synchytrium* entsprechend, wo ebenfals die allein vorhanden Dauersporen bei der Keimung einen Sporangiensorus liefern." Schroeter (**1897**) failed to mention *Micromyces* in his monograph on the Chytridineae, but de Wildeman (**1900**) expressed the same viewpoint as Fischer regarding the taxonomy of the genus. Its fundamental similarity to *Synchytrium* was again

emphasized by Scherffel (**1926**) who stated, "Die Übereinstimmung zwischen einem *Pyknochytrium* und *Micromyces* ist überhaupt eine derart vollkommene, dass es sich fragt, ob die weitere Aufrechterhaltung der Gattung *Micromyces* zulassig ist." Couch (**1931**) likewise pointed out that the life cycle, development and cytology of *Micromyces* species are strikingly like those of *Synchytrium* species whose sporangial sori develop as an extrusion or outgrowth of the mature thallus or initial cell (*Mesochytrium*). To quote Couch (**1931**) who gave an adequate comparison of the two genera as they were known at that time:

"In comparison with *Micromyces* the prosori and resting bodies of *Synchytrium* are much larger, reaching a maximum size in the former genus of 33.6 μ and in the latter of 180 μ or more. In *Synchytrium* the prosori and resting spores are seldom if ever spiny. The resting spores, however, are often covered with a thick warted membrane, or the membrane may be coarsely reticulated as in *S. endobioticum* or *S. saxifragae*. The prosorus contains a single large nucleus which in *S. endobioticum* attains the enormous diameter of 25 μ. The nucleus in the prosorus of the present species of *Micromyces* is 8 μ thick, a comparatively large nucleus for such a small thallus. In the subgenus *Mesochytrium* a thin-walled vesicle is extruded from the prosorus and the protoplasm flows into this and is then delimited into sporangia. In *Micromyces zygogonii* the sorus is formed in precisely the same way. The number of sporangia contained in one sorus in *Synchytrium* is often up to 100 or even more, rarely 5–10 (*S. endobioticum*). In *Micromyces* the number varies from 3–24. The swarmspores are uniciliate and vary from 3–6 μ thick; in *Micromyces* they are about 1 μ thick. Curtis (**1921**) has shown that in addition to swarmspores gametes also are formed which fuse in pairs to form a biciliate zygote and from this zygote the resting spore is formed. The gametes except for their behavior are indistinguishable from the swarmspores. In the species of *Micromyces* here described I have found an apparent fusion of gametes similar to that described by Curtis. In short the cytology and development of the present species of *Micromyces* and *Synchytrium* as illustrated by *S. endobioticum* are strikingly similar. But in view of the slight morphological differences between the two genera and the great difference in size correlated with a striking difference in habitat it seems best to retain the genus *Micromyces* rather than merge it with the sub-genus *Mesochytrium*."

The species referred to above as *M. zygogonii* was later found to be a different species which Couch (**1937**) named *M. longispinosus*.

Another difference, which was not emphasized by Couch, is that in *Micromyces* the young newly-entered parasite may be amoeboid in movement and develop pseudopodia which are frequently retracted and formed anew (Canter, **1949**). Such changes in shape and motion have not been reported for *Synchytrium*, although young thalli apparently move or are moved to the base of the host cell as in *S. endobioticum*. Also, in fixed and stained preparations they may appear somewhat irregular in outline and lie close to the host nucleus as in species of *Micromyces*. *Micromyces* apparently does not form a common and distinct sorus membrane which independently envelops all of the sporangia as in *Synchytrium*. The descriptions of most students of *Micromyces* are not certain on this point, and their drawings do not indicate that the sporangia are liberated by the rupture of a separate sorus wall. However, in *M. ovalis* Rieth (**1950B**) has figured and described the sorus membrane as rupturing or breaking only at the points of contact of the four cleavage segments or sporangia. The four segments of the sorus membrane become, thus, the outer wall of the sporangia.

However, this difference, together with the amoeboid movement of the thallus in *Micromyces*, differences in size, spinyness of the resting spores, habitat and hosts are not generically significant in the author's opinion. They are outweighed by the fundamental structural and developmental similarities of the two genera. There are no valid reasons, so far as our knowledge goes at present, for retaining *Micromyces* as a separate genus, and it is herewith merged with *Synchytrium*. Its species are accordingly renamed **S. zygogonii** (Dang.) comb. nov., **S. petersenii** (Scherff.) comb. nov., **S. spirogyrae** (Skvort.) comb. nov., **S. longispinosus** (Couch) comb. nov., **S. laevis** (Canter) comb. nov., and **S. ovalis** (Rieth) comb. nov. De Wildeman's (**1900**) *Micromyces mesocarpi* is obviously a species of *Micromycopsis* and need not be considered here.

Couch emphasized the close resemblance of *Micromyces* to the subgenus *Mesochytrium* of which *S. endobioticum* is regarded as a typical member. However, in this species the resting spore func-

tions as a sporangium in germination and gives rise directly to zoospores. In the former *Micromyces* species (*S. zygogonii*, *S. longispinosus* and *S. laevis*) the resting spore functions as a prosorus in germination by extruding a thin-walled vesicle into which the protoplasm flows and later segments into sporangia. Therefore, on this basis they do not belong in *Mesochytrium* or any of the other known subgenera. They have the same life cycle and sequence of development as *S. fulgens*, with the exception that in the latter species the mature thallus functions as a sorus. Fusion of motile isogametes to form a zygote has been reported in both species, although the present-day evidence of sexuality in *S. longispinosus* is not as convincing as for *S. fulgens*.

In the latter species sexual reproduction occurs so generally that Kusano considered it to be typical and asexual reproduction as accessory. In *S. longispinosus*, on the other hand, only fusing gametes and biflagellate zygotes, which later penetrated the host cell, have been observed. Couch (**1931**) was unable to follow the development of the zygotes into mature resting spores.

In view of the fact that former *Micromyces* species do not fit into any of the known subgenera a new one, *Microsynchytrium*, is proposed to include them. This name was chosen because the species included in the subgenus at present are very minute in comparison with most members of the other subgenera. Germination of the resting spores in *S. spirogyrae*, *S. petersenii* and *S. ovalis* has not been observed, and their inclusion in *Microsynchytrium* at present is open to question.

Furthermore, in light of Kusano's (**1930**) intensive study of *S. fulgens* and discovery that the resting spores function as prosori in germination it is obvious that this species also does not belong to any of the known subgenera. Accordingly, another subgenus, *Exosynchytrium*, is proposed for it and other species which may be found in the future to have the same life cycle and type of development. This subgenus is placed near *Eusynchytrium* in the same sense that *Microsynchytrium* is close to *Mesochytrium*. It is quite probable that other known species of *Synchytrium* will fit into either of these subgenera when their full life cycles become known.

Two other genera of aquatic algal parasites, *Micromycopsis* and *Endodesmidium*, are included in the Synchytriaceae and appear to be closely related to *Synchytrium*. However, their life cycles are not fully known, and it is impossible to determine the degree of relationship at the present time. The former genus was erected by Scherffel (**1926**) for two species, *M. cristata* and *M. fischeri*, which parasitize *Hyalotheca dubia* and *Zygogonium* sp. in Hungary. Subsequent studies by Sparrow (**1932**), Cejp (**1932**, **1933**), and Canter (**1949**) indicated a wider distribution of these species as well as the presence of other species in algae. Canter's excellent study in particular has broadened our concepts of the aquatic Synchytriaceae. She completed our knowledge of the life cycle of Scherffel's *M. fischeri* and described two new species, *M. intermedia* and *M. mirabilis* in England. Thus, *Micromycopsis* at present includes five species and one variety.

It differs from the aquatic (*Micromyces*) species of *Synchytrium* in that the prosorus develops an exit tube or canal of variable length through which its protoplast flows to the outside of the host cell and forms an epibiotic sporangial sorus. Occasionally, however, the latter may develop endobiotically without the formation of an exit canal as in the former *Micromyces* species. Also, it should be noted here that *S.* (*Micromyces*) *ovalis* rarely develops an exit canal as in *Micromycopsis* species, which apparently indicates a close relationship. Another difference noted occasionally by Scherffel in *M. cristata* and confirmed by Canter for *M. fischeri* is that the uniflagellate zoospores from the sporangia may exhibit only jerky or amoeboid movements after which they soon become quiescent and round up. Their protoplasm then undergoes cleavage into segments which develop into minute secondary uniflagellate zoospores and swim actively away. Accordingly, *Micromycopsis* develops primary and secondary sporangial phases and produces both primary and secondary zoospores as well.

Endodesmidium was created by Canter (**1949**) for an endobiotic parasite of *Netrium oblongum*, *Cylindrocystis crassa* and *C. brevissonii*. It produces a prosorus which germinates when mature and gives rise to a sessile endobiotic sorus. The latter develops two lateral exit papillae which may or may not pierce the host cell wall.

Its protoplasm cleaves into about fifty globular segments which ooze out singly in succession through the papillae, either into the external medium or into the cavity of the host cell. These bodies rarely develop a posterior flagellum but usually they are sluggish or remain quiescent, or undergo amoeboid movement. They are potential sporangia and soon become quiescent and round up. Subsequently, their content cleaves into 2 to 5 minutes posteriorly uniflagellate zoospores as noted occasionally in *Micromycopsis*. On one hand, *Endodesmidium* appears to have only one sporangial phase in the development of which the potential sporangia are discharged through exit papillae from the sorus and may behave for a short time as rudimentary primary zoospores. If, on the other hand, the discharged potential sporangia actually become motile and behave like zoospores, the entire sorus might be regarded as a primary sporangium.

Resting spores have not been observed in *Micromycopsis* and *Endodesmidium*. Therefore, it is not known whether they function directly as zoosporangia (*S. endobioticum*) or as prosori (*S. zygogonii*, etc.) in germination. Nevertheless, the development of exit canals by the prosorus (*Micromycopsis*) and papillae by the sorus (*Endodesmidium*) as well as the presence of primary and secondary zoospores are unknown in *Synchytrium*, and these are the principal differences which separate the genera at present. It is not altogether improbable that secondary zoospores may be found in species of *Synchytrium* also when they have been studied more carefully, and this is a developmental phase which should be searched for and investigated intensively.

In this connection it should be noted again that *Micromycopsis fischeri* occasionally forms an endobiotic sessile sorus without developing an exit canal. Such thalli are almost indistinguishable from those of the former *Micromyces* species. For this and other reasons Canter believes that ". . . it is clear that in the near future *Micromyces* and *Micromycopsis* may have to be merged into one genus." Rieth's (**1950B**) recent discovery that *S. (Micromyces) ovalis* rarely forms an exit canal lends support to Canter's view, or at least indicates that the two genera are closely related. However, so far as our knowledge goes, *Micromyces* usually has the same life

cycle as some species of *Synchytrium*, and these algal parasites may possibly represent a transition series from *Endodesmidium* and *Micromycopsis* to the terrestrial species of *Synchytrium*. Canter regards *Endodesmidum* as the most primitive genus of the Synchytriaceae. From this genus through *Micromycopsis fischeri*, *M. cristata* and *M. zygnaemicola* in succession the secondary sporangial phase is gradually suppressed until it is lacking in the aquatic (*Micromyces*) and terrestrial species of *Synchytrium*.

Classification of all *Synchytrium* species in subgenera is impossible at present because so few of them are fully known. Of the 158 or more species reported in the literature less than 25 are completely known and can be classified with certainty. Unfortunately, most studies on *Synchytrium* during the last three decades or more have been taxonomic and very little attention has been given to life cycles, developmental sequences and host range. As a result the majority of species are known only in their sorus, or prosorus, or resting spore stages. Other more recent studies (Cook, **1945–53**) have been concerned primarily with the type of galls produced on the host plants as a possible aid in classification. Unfortunately, many of the fungi were neglected so much in these studies that it is impossible in some cases to determine whether the investigator was describing prosori, or sori, or resting spores, and many of the new species created by Cook will have to be reinvestigated thoroughly before they can be properly classified. The same is true of the new species reported by Mhatre and Mundkur (**1945**), Patel, Kulkarni and Dhande (**1949**),[1] Padwick (**1945**), Lacy (**1950**), Ramakrishnan (**1950**) and Payak (**1951**).

The type of gall produced is helpful in distinguishing species, but it is not always definitely indicative of the type of life cycle and development. Furthermore, galls bearing sori or prosori may be quite different from those which bear the resting spores, *i.e. S. pulverum*. In most cases gall characteristics can be of only secondary value in taxonomy.

[1] The name *S. phaseoli* has already been given to a species by Weston (**1930**). Accordingly, their use of this binomial is invalid, and I am proposing the name **S. indicum** (Patel, Kulk. & Dh.) comb. nov. for their species.

As noted above, very few species are completely known as far as life cycle and development are concerned. Those which are fully known may be grouped into six subgenera as indicated in the key below.

KEY TO THE SUBGENERA OF SYNCHYTRIUM

I. Resting spores unknown. Mature thallus functioning directly as a summer sorus of thin-walled sporangia; sporangia delimited by cleavage within the soral wall, freed by its rupture, and appearing as powdery masses in open aecidium-like pustules.....Subgenus *Woroninella*

II. Resting spores known

A. Life cycle including summer sporangial sori and resting spores.

1. Mature thallus functioning directly as a sorus of thin-walled sporangia; sporangia delimited by cleavage within the sorus wall and freed by its rupture.

a. Resting spore functioning as a sporangium in germination and giving rise directly to zoospores.....Subgenus *Eusynchytrium*

b. Resting spore functioning as a prosorus in germination; content emerging to form a thin-walled sorus which cleaves into sporangia.....Subgenus *Exosynchytrium*

2. Mature thallus functioning as a prosorus; content emerging from initial cell to form a thin-walled sorus which cleaves into sporangia.

a. Resting spore functioning as a sporangium in germination and giving rise directly to zoospores.....Subgenus *Mesochytrium*

b. Resting spore functioning as a prosorus in germination; content emerging to form a thin-walled sorus which cleaves into sporangia.....Subgenus *Microsynchytrium*

B. Life cycle including only resting spores; summer sporangial sori unknown.

1. Resting spore functioning as a prosorus in germination.

a. Protoplast emerging to form a thin-walled sorus which cleaves into sporangia.....Subgenus *Pycnochytrium*

2. Resting spore functioning as a sorus in germination.

a. Protoplast not emerging; cleaving directly into several sporangia which dehisce irregularly with the rupture of the resting spore wall.....Subgenus *Endosynchytrium*

In 1933 DuPlessis added the subgenus *Endochytrium* as a provision for his *S. cotulae*, but inasmuch as this name had already been used by Sparrow (**1933**) for another chytrid genus it is invalid. Accordingly, the author (**1941**) proposed the name *Endosynchytrium* for this subgenus.

This key is an extension and modification of the one proposed by Fitzpatrick (**1930**), and is based on the presence or absence of summer sporangial sori, or prosori and resting spores, the type of resting spore germination, as well as on the development of the

mature vegetative thallus into a sorus or its function as a prosorus. The general noncommittal term resting spore is used for the relatively dormant, thick-walled stage, regardless of whether it functions as a prosorus, or sorus, or a sporangium in germination, or whether or not it is formed as the result of fusion of motile isogametes. Also, no distinction is made between sporangia and gametangia or between zoospores and gametes because they are morphologically and structurally alike so far as is known at present. Sexual reproduction has been reported in only *S. endobioticum*, *S. fulgens*, and *S. longispinosus*, and it is difficult to incorporate distinctions on this basis at present in a general key to all species of *Synchytrium*. Further division of the subgenera into groups such as *Leucochytrium* and *Chrysochytrium* on the basis of color is not as significant taxonomically as it was believed to be. Color of the thallus is not a reliable criterion because it varies markedly with age. Thalli may be almost hyaline or lemon-yellow when young and gradually become dark yellow to deep orange with maturity. Obviously, any key is only provisional, and the proposed one will doubtless have to be modified markedly as old species become better known and new ones are added to the genus.

On the basis of present-day knowledge the following reported species may be assigned provisionally to the various subgenera listed above.

WORONINELLA: *S. aecidioides*, *S. minutum*, *S. citrinum*, *S. psophocarpi*, *S. vulcanicum*, *S. dolichi*, *S. atylosiae*, *S. vignicola*, *S. aequatoriensis*, *S. phaseoli* and *S. crotulariae*.

EUSYNCHYTRIUM: *S. taraxaci*. In *S. papillatum*, *S. sanguineum*, *S. geranii*, *S. andinum*, *S. amsinckiae*, *S. trichophilum*, *S. uliginicola*, *S. cellulare*, *S. australe*, *S. valerianellae* and *S. linderniae* both sporangial sori and resting spores have been reported, but germination of the latter has not been observed. Therefore, it is not certain that they belong in *Eusynchytrium*.

EXOSYNCHYTRIUM: *S. fulgens*.

MESOCHYTRIUM: *S. endobioticum*. Usually, *S. succisae* and *S. stellariae* are included in this subgenus, but whether or not they belong here will not be certain until germination of their resting spores has been observed.

MICROSYNCHYTRIUM: *S. zygogonii, S. longispinosus,* and *S. laevis.* Whether or not *S. spirogyrae, S. petersenii* and *S. ovalis* belong in this subgenus will not be certain until germination of their resting spores has been observed.

PYCNOCHYTRIUM: *S. myosotidis, S. luteum, S. punctatum, S. punctum, S. pilificum, S. potentillae, S. fuscus, S. mercurialis, S. globosum, S. rubrocinctum, S. pyriforme, S. musicola* (?), *S. alpinum, S. scirpi, S. aureum, S. ulmariae, S. auranticum, S. anemones, S. anomalum, S. alpinum,* etc.

ENDOSYNCHYTRIUM: *S. cotulae.*

The remaining 80 or more species of *Synchytrium* are too poorly known to be classified with any degree of certainty in the above subgenera. It is obvious that an immense amount of research remains to be done on *Synchytrium,* and it is hoped that present and future investigators will study the individual species more intensively instead of making new species on incomplete and fragmentary evidence.

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SOME NEW SPECIES OF SYNCHYTRIUM FROM BANARAS

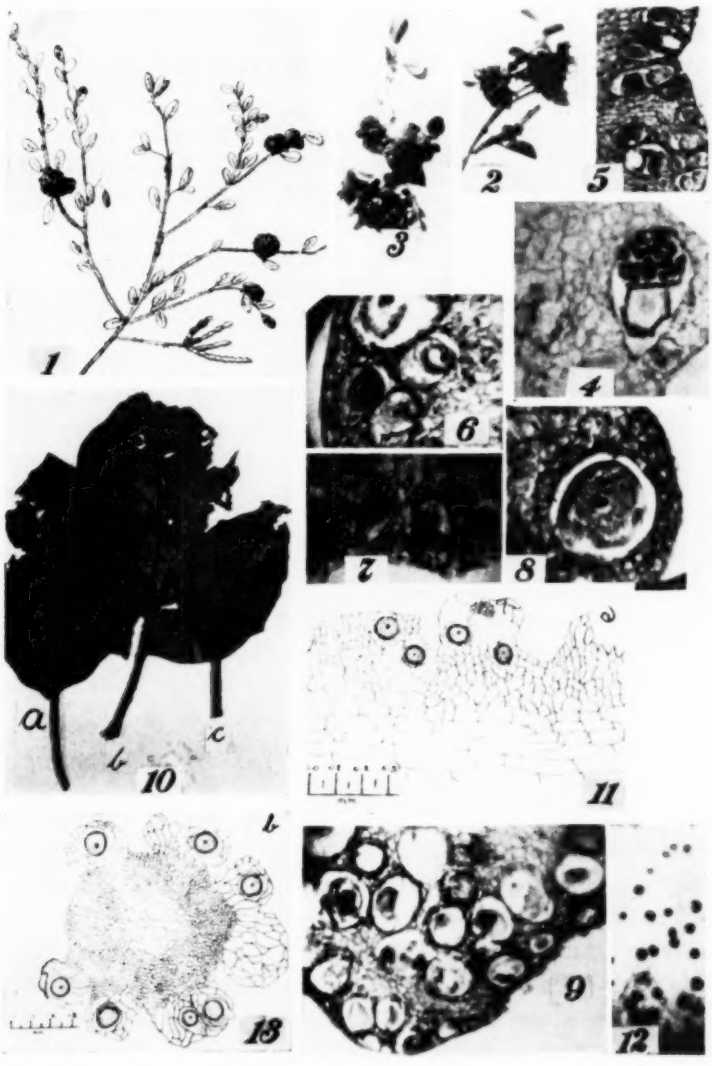
B. T. Lingappa

(with 13 figures)

A SYNCHYTRIUM CAUSING A WART DISEASE OF ALYSICARPUS MONILIFER DC.

This interesting new species was found by the author at Banaras, India. The host plant is a moderately spreading perennial weed very common in the lawns and along the road sides. All aerial parts die out during summer but make a fresh growth with the coming of rains in September. All the aerial parts, leaves, petioles, stems, fruits, and especially the axillary and spical buds become infected (Fig. 1). The infected parts produce generations of summer sori, and this leads to extensive proliferation of the tissue and results in the formation of warts. These warts are conspicuous at the close of the season (Figs. 2, 3). They are light green to yellow and later become brown. They show different stages of prosori in the earlier stages and the hypnospores dominate as the season advances. Sometimes, fast developing tender shoots become severely infected and develop into a whorl of prominent wart-aggregates at the crown. At the close of the season the warts are full of hypnospores which dry up, crumble and are mixed with the soil. During the next rainy season the new branches develop and come in close contact with the soil, due to their creeping habit. The tender parts are thus easily accessible to the zoospores and the disease is carried on. Wet rot of the warts does not occur.

The galls on the leaves are minute, spherical or cupulate and yellow. Galls containing prosori rupture, become cupulate and release zoosporangia. Leaves suffering from the production of many generations of summer sori become much distorted and yellow. Galls produced later contain hypnospores, are simple, much smaller and produce less distortion.



FIGS. 1–13.

The warts produced in this case are very similar to those of the potato wart in morphology and development. All the warts are aerial. The sequence of development of this fungus is also similar to *S. endobioticum* (Schilb.) Perc. The zoospores gain entrance into the epidermal cells of the host. Almost every other epidermal cell may be infected (FIG. 5). Multiple infections are very common (FIGS. 5, 7). More than three fungus bodies were frequently seen developing in a single cell. Soon after entry they become spherical but may become elongated when more than two are growing in a cell. The zoospores enter the host cell without leaving any path of entry. The nuclear structure attains its maximum size very early (FIG. 6). The nucleolus lies in the center of the nuclear vacuole and is deeply stained. The fungus at maturity has a thin yellow exospore and much thinner hyaline endospore. It develops into a prosorus. The contents of the prosorus flow into an extruded thin vesicle. The nucleus also passes into it and undergoes numerous divisions. Cleavage planes appear, breaking the whole mass into uninucleate polyhedral pieces which develop into sporangia. The vesicle is extruded, not through a small slit in the exospore as reported in the formation of prosori in other *Synchytrium* species, but is formed by an elongated rupture of the exospore (FIG. 4). The zoosporangia are pyriform or oval, yellow, and undergo much distension causing the rupture of the galls. The exospore of the prosorus, which is much shrivelled, thin, dirty yellow, and boat-shaped, may remain behind the cupulate galls.

Later in the season, the warts are full of thick-walled hypnospores. It is only in the cells containing the mature hypnospores that the cell contents precipitate as a brown mass. But the contents of the host cells in which prosori are developing are not so affected even up to the time of their dehiscence. The host nucleus, however, disintegrates very early in both cases. Though multiple infections were so common, the development of more than one hypnospore is rare and invariably only one prosorus develops in a single host cell. Differences in the age of the developing fungus bodies were common (FIG. 7). It may be possible that in cases of multiple infections only one of them will ultimately develop while others become stunted and disappear later.

Synchytrium cookii sp. nov.

Gallae minutae, luteae, sphericae, in utraque pagina foliorum. Gallae cupulatae plus aggregatae quam gallae sphericae, 0.2–0.3 mm. diam., distortionem partium infectarum causantes. Alabastra axillaria affectata atque partes juniores culmorum evolvuntur in excrescentias verruculosas 2 cm. diam., vel largiores, luteas, postea brunneas. Cellulae epidermales infectae. Infectio multiplex communis est. Ut plurimum unus prosorus vel una hypnospora in una cellula evolvitur. Prosorus exosporio tenui, luteo et endosporio hyalino ornatus. Sporangia 50–70, spherica vel pyriformia, lutea, in vesiculo extruso delimitata, 20–22–26 μ diam. Hypnosporae plurimae, singulae in cellulis epidermalibus, parietibus crassis praeditae, dense luteae, sphericae vel ellipsoideae, contentis luteis granularibus, 50–85–120 μ diam. Exosporium 5 μ crassum.

Typus afficiens alabastra axillaria, folia atque culmos *Hysicarpi moniliferi* DC. a B. T. Lingappa inventus, in loco Banaras, Indiae, die 12 septembris 1950.

Galls minute, yellow, spherical, on both surfaces of the leaves, also on axillary buds and stems. Cupulate galls more crowded than the spherical galls, measuring 0.2 to 0.3 mm. in diameter, causing distortions of the affected parts. Affected axillary buds and tender parts of the stem developing conspicuous warty outgrowths, measuring 2 centimeters or more in diameter, yellow, becoming brown. Epidermal cells infected. Multiple infection common. Commonly one prosorus or a hypnospore develops in a single cell. Prosorus with a thin yellow exospore and a hyaline endospore. Sporangia 50 to 70 in number, spherical to pyriform, yellow, delimited inside the extruded vesicle, 20–26 μ (av. 22 μ) in diameter. Hypnospores numerous, one inside an epidermal cell, thick-walled, deep yellow, spherical or ellipsoid, with yellow granular contents, measuring 50–120 μ (av. 85 μ) in diameter. Exospore 5 μ thick.

A SYNCHYTRIUM ON BRINJAL PLANTS (SOLANUM MELONGENA)

A species of *Synchytrium* was found attacking Brinjal plants near the Hindu University in 1950 and again in September 1951. The fungus attacks leaves and other tender parts. This is followed by the formation of simple or aggregated galls on both surfaces (FIG. 10) which are very abundant near the tips and along veins. Infections are also severe on petioles and stems, which become encrusted and irregularly thickened. They also become crustaceous on the midribs. They are brown and thickened. The distortions of the leaves are not severe, but the leaves are thicker than usual.

The galls are spherical at first but become cupulate with the dehiscence of the prosori. Such erumpent galls may coalesce. The hypnospores are produced in much smaller galls. The galls are yellow, becoming dirty brown. The cupulate galls are 0.4–1 mm. in diameter, while the galls containing the hypnospores are up to 0.2 mm. in diameter. Each gall may contain three or more hypnospores but only one hypnospore in a cell (FIG. 11). The summer spores become prosori. The sporangia are 30 to 40 in number, yellow, spherical at time of dehiscence and become pyriform or beaked later. Zoosporangia at time of release from vesicle measure 16–18 μ in diameter. Hypnospores spherical or oval, dark brown, with 6–9 μ thick brown exospore. The disintegrated host cell contents form a third wall over the hypnospore. Infections spread from nursery beds and the seedlings raised during heavy rains are attacked most severely. The disease under such conditions spreads rapidly to the growing stem apex and the seedlings so infected are useless for transplanting. Other records on Solanaceous hosts differ much from this fungus which is a member of the subgenus *Mesochytrium*.

Synchytrium akshaiberi sp. nov.

Gallae minutae, sphericae, leves, nitentes, luteae vel brunneae, per foliorum paginas dispersae, 90–250 μ diam. Gallae cupulatae largiores, haud altae, ovatae, crustaceae, 0.8–1.5 mm. diam., plus aggregatae in foliorum petiolis, culmis atque nervis mediis incrassationes irregulares causantes, luteae, postea brunneae. Prosori lutei, parietibus crassis ornati. Sporangia 30–40, ovalia, lutea, 16–18 μ diam., delimitata in vesiculo extruso. Hypnospora una vel plures in singulis gallis, singulae in cellulis plantae hospitis, dense brunneae, parietibus crassis ornatae, sphericae, raro oblongae, 48–92–130 μ diam., contentis luteis granularibus. Exosporium 6–9 μ crassum.

Typus in foliis, petiolis atque culmis *Solani melongenae* L., in loco Banaras, India, die 21 septembris, 1950, a B. T. Lingappa inventus.

Galls minute, spherical, smooth, glistening, yellow to brown, studded over the leaf surfaces, measuring 90–250 μ in diameter. The cupulate galls are bigger, shallow, oval, crustaceous, measuring 0.8–1.5 mm. in diameter, crowded on the petioles, stem and midribs causing irregular thickenings, yellow, later turning brown. Yellow, thin-walled prosori formed. Sporangia 30–40 in number, oval, yellow, measuring 16–18 μ in diameter, delimited within an extruded vesicle. Hypnospores one or more in a gall, one inside

a single host cell, dark brown, thick-walled, spherical, rarely oblong, measuring 48–130 μ (av. 92 μ) in diameter, with yellow granular contents. Exospore 6–9 μ thick.

A SYNCHYTRIUM ON MELILOTUS

The fungus attacks *Melilotus indica*, a very common weed in the rainy season. The disease appears on all parts of the plants for a very short period and causes extensive defoliation. The stems and branches often show crowded patches of spherical yellow galls which turn dark brown later. The infected leaves are distorted, turn yellow and are soon shed. The life-history of the fungus is very short. After a long period of exposure to rains the dormant hypnospores in the soil release zoospores which invade the epidermal cells of the host and develop directly into big yellowish brown hypnospores. The galls are simple, yellow, becoming brown; each of them contains one to four hypnospores. But only one hypnospore occurs in a single host cell. At the maturity of the hypnospores, disintegrated, brown contents of the host cells are deposited on the exospores as a third layer. This species is a *Chrysochytrium* of the subgenus *Pycnochytrium*. It differs markedly from the two above-mentioned species of the subgenus *Mesochytrium* in having a very short life-cycle. The disease appears quite late in the season; possibly the hypnospores have a prolonged period of dormancy and therefore germinate after a prolonged period of exposure to rains. The fungus is insured against the possibility of complete destruction by the rapid shedding of the affected leaves soon after the hypnospores have matured.

Synchytrium meliloti sp. nov.

Gallae prominentes, sphericae, leves, pallide luteae, 0.2–0.4 mm. diam., in petiolis, culmorum partibus atque in utraque pagina foliorum aggregatae. Folia affectata distorquentur, aliae partes tenuiter incrassantur, brunneae. Sporangia nulla observata. Hypnosporae largae, sphericae, pallide brunneae, una tantum vel plures tribus in una galla, una in cellula epidermali, 100–120–165 μ diam., contentis luteis, minute granularibus.

Typus in foliis atque culmis *Meliloti indicae* All. in loco Banaras, India, die 25 septembris 1951, a B. T. Lingappa inventus.

Galls prominent, spherical, smooth, light yellow, measuring 0.2 to 0.4 mm. in diameter, crowded on petioles and stem parts and on

both surfaces of the leaves. Infected leaves distorted; other parts slightly thickened and turning brown. No sporangial formation observed. Hypnospores large, spherical, light brown, one or rarely more than three in a gall; one in an epidermal cell; 100–165 μ (av. 120 μ) in diameter, with finely granular yellow contents.

In proposing the above three species as new, the author has closely followed the latest trend of work in this genus (**3**). Field observations and frequent examinations of fresh collections have facilitated an understanding of the true nature of these species.

The type specimens of the above species will be deposited at the Herb. Crypt. Orient. New Delhi. The author is highly thankful to his professor Dr. Akshaiber Lal for the encouragement and facilities; to Rev. Father Dr. H. Santapau for translating the diagnoses into Latin, and to the officers of the Herbarium at I.A.R.I. New Delhi for providing facilities to examine the collections available there. Thanks are due to Mr. D. L. Bohra, college artist, for photomicrographs. The author is much indebted to Drs. M. T. Cook, B. B. Mundkur and Robert Page, for reprints. Dr. M. T. Cook has kindly gone through the paper and made valuable corrections for which the author is much indebted.

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EXPLANATION OF FIGURES

FIGS. 1–9. *Synchytrium cookii*. 1. A portion of *Alysicarpus monilifer* showing symptoms of infection. 2. Warts of axillary buds and stem. 3. Warts of the stem apex. 4. Zoosporangia formed in the extruded vesicle. Empty case of the prosorus still intact with the vesicle. Vesicle extruded through the wide opening. 5. Severe infection of the epidermal cells, showing multiple infection. 6. Prominent nucleolus and nucleate vacuole in all the

three very young stages of the fungus bodies. 7. Multiple infection showing differences in age of the developing fungus bodies. 8. Young developing prosorus, highly enlarged. 9. Showing the distribution of hypnospores within the wart tissue.

FIGS. 10—11. *Synchytrium aksheiberi*. 10. Brinjal leaves showing distribution of galls. Characteristic symptom on petioles and midribs as compared to petiole of 'a.' 11. Section through an affected leaf showing the galls, cupulate gall hypnospores and zoosporangia in extruded vesicle. Portion of cupulate gall.

FIGS. 12–13. *Synchytrium meliloti*. 12. Enlarged view of the hypnospores in the galls and their distribution on the leaf surface. × 24. 13. Section through affected stem showing nature and distribution of galls and hypnospores.

THE NEW RULES OF TYPIFICATION AS THEY AFFECT SARCOSCYPHA AND VELUTARIA

RICHARD P. KORF

The new rules covering the selection of a type (Lanjouw **1951**) will serve to answer many perplexing situations in botanical nomenclature. Two nomenclatural problems in the Discomycetes have puzzled me for some time. The rules, as written, provide a not too laborious solution to these problems. The result of one of these is encouraging, in that it allows the use of names long current in the literature and well-known even to those with but a cursory interest in the cup-fungi. The result of the other, however, is displeasing in that it means using a well-known name in a sense in which the name has rarely been employed, and may leave another group of species without an available generic name.

The two problems outlined below have certain points in common: the genera were erected originally with two species each, and in neither case was a type designated. Neither the new Art. 19 nor Recommendation V (4e) will apply, as it is not possible to say in either case that one of the species fits the generic diagnosis better than the other. Article 18, Note 3 seems to decide the issue in both cases:

"If no holotype has been indicated by the author who described a taxon, or when the holotype is lost or destroyed, a substitute for it must be chosen. The author who makes this choice must be followed unless it can be proved that the choice was not made in accordance with Art. 19. The substitute may be either a *lectotype* or a *neotype*. A lectotype always takes precedence over a neotype.

"A *lectotype* is a specimen or other element selected from the *original material* to serve as nomenclatural type when the holotype was not designated at the time of publication or so long as it is missing.

"When two or more specimens have been designated as types by the author of a name (i.e., male and female, flowering and fruiting, etc.) one of them must be chosen as *lectotype*.

"A *neotype* is a specimen selected to serve as nomenclatural type so long as all of the material on which the name of the taxon was based is missing."

PLECTANIA, SARCOSCYPHA, AND RHIZOPODELLA

The common scarlet-cup of the early spring has passed for many years under the name *Sarcoscypha coccinea*. This nomenclature was overturned when Seaver (**1928**) revived the name *Plectania coccinea* for the fungus. Recent authors have followed Seaver, notably Kanouse (**1948**) and Nannfeldt (**1949**). The discovery of different types of spore germination in this species and in what is presumably *S. coccinea* var. *jurana* Boud. (Rosinski **1953**) led to an investigation of the correct generic name to apply to the scarlet-cups.

Plectania was erected by Fuckel in 1870 to include two species, *P. coccinea* (Scop. ex Fr.) Fckl. and *P. melastoma* (Sow. ex Fr.) Fckl. One of these species must be designated the lectotype.

Boudier (**1885**) founded two genera containing these species: *Sarcoscypha* (Fr.) Boud., based on *Peziza coccinea* Scop. ex Fr. and *P. occidentalis* Schw.,[1] and *Rhizopodella* (Cooke) Boud., with *Peziza melastoma* Sow. ex Fr. designated the type. Depending upon the choice of a lectotype for *Plectania* Fuckel, one or the other of Boudier's genera must be considered a synonym of *Plectania*.

Saccardo (**1889**) was apparently the first author to divide the genus *Plectania* into two. Inasmuch as the genus originally contained only two species, this division automatically selected a lectotype. He recognized two genera for the species involved: *Plectania* Fckl., including *P. melastoma* and a number of additional species, and *Sarcoscypha* (Fries) Boudier (as "Fries"), embracing *S. coccinea* and certain other species.

When the new rules of typification are applied, which consider the principle of priority, it is clear that it is necessary to return to the generic name *Sarcoscypha* for the scarlet-cups, instead of using

[1] The latter species is still considered congeneric with *P. coccinea* in all recent works known to me. It was not treated by Fries, however, and is therefore not eligible as the type of the Fries subgenus. The only other species known to Fries and included by Boudier in the genus is *P. coccinea*, which must be considered as the lectotype of the genus.

Plectania as some recent authors have done. Further, the correct generic name for *Peziza melastoma* is *Plectania*, a name by which it has long been known, and not *Rhizopodella*, as used by Kanouse (**1947**, **1948**), Nannfeldt (**1949**) and Korf (**1949**). The synonymy and typification of the genera is therefore as follows:

PLECTANIA Fuckel, Symb. myc. 323. [1870.], *emend.* Sacc. Syll. Fung. **8**: 163. 1889.

SYNONYMS: *Peziza* ser. *Lachnea* subgen. *Rhizopodella* Cooke, Mycogr. **1**: 260. 1879. [Lectotype: *Peziza melastoma* Sow. ex Fr., designated by Boudier (**1885**).]

Peziza subgen. *Plectania* (Fckl.) Sacc. Bot. Centralbl. **18**: 215. 1884. (Holotype: *Peziza melastoma* Sow. ex Fr.)

Rhizopodella (Cooke) Boud. Bull. Soc. Myc. France **1**: 103. 1885. [Holotype: *Peziza melastoma* Sow. ex Fr. (*Rhizopodella melastoma* (Sow. ex Fr.) Richon, Catalogue raisonné des Champ. 191. 1889.)]

TYPE SPECIES: *Peziza melastoma* Sow. ex Fr. (*Plectania melastoma* (Sow. ex Fr.) Fckl. Symb. myc. 324. [1870.]), effectively selected by Saccardo (**1884**, **1889**).

SARCOSCYPHA (Fries) Boudier, Bull. Soc. Myc. France **1**: 103. 1885.

SYNONYM: *Peziza* ser. *Lachnea* trib. *Sarcoscyphae* Fries, Syst. myc. **2**: 78. 1822. [Lectotype: *Peziza coccinea* Scop. ex Fr. Syst. myc. **2**: 79. 1822, effectively selected by Boudier (**1885**).]

TYPE SPECIES: *Peziza coccinea* Scop. ex Fr. (*Sarcoscypha coccinea* (Scop. ex Fr.) Lambotte, Fl. myc. belg., suppl. 1, 302. 1887) effectively selected by Boudier (**1885**).

Note that the combination *S. coccinea* is usually erroneously attributed to Saccardo rather than to Lambotte.

VELUTARIA, TAPESINA, ETC.

The genus *Velutaria* was erected by Fuckel in 1870, and included two species, *V. rufo-olivacea* (Alb. & Schw. ex Fr.) Fckl. and *V. grisco-vitellina* (Fckl.) Fckl. In 1887,[2] the genus *Tapesina* was founded by Lambotte, and likewise included two species, *T. ruborum* (Cooke & Phil.) Lambotte and *T. retincola* (Rabenh.) Lambotte. The latter species is today considered a true *Tapesia*. The former

[2] In my monograph of the Arachnopezizeae (Korf **1951**), I gave the date 1888 for Lambotte's paper. This appears to be in error, as the separates of this work were dated (and presumably issued in) 1887, while the volume of the Mém. Soc. Roy. Sc., Liège, in which the work was published, appeared in 1888. The citations in my paper (*loc. cit.*, pp. 150, 151) should therefore read: Fl. myc. belg., suppl. 1 (same pages), 1887.

is a synonym of *Peziza grisco-vitellina* Fckl., and treated as the type of *Tapesina* under the name *T. grisco-vitellina* (Fckl.) Höhnel. It is so used by von Höhnel (**1923**), Nannfeldt (**1932**), Kanouse (**1947**), Dennis (**1949**), Gremmen (**1951**) and Korf (**1951**).

The generic name *Velutaria* has come to be applied to *V. rufo-olivacea* and its allies. It is treated thus by Rehm (**1892**), Lindau (**1897**), Nannfeldt (**1932**), Kanouse (**1947**), Petrak (**1949**), Gremmen (**1951**), Groves (**1951**) and Seaver (**1951**). Such unanimity of opinion is rarely reached in discomycete nomenclature. A strict application of the new rules of typification will, however, completely disrupt this harmony, upsetting the very stability of nomenclature which the rules are intended to supply.

The case is not unlike that of *Plectania*. Again Saccardo was apparently the first to divide the genus. In 1884 he emended the genus, designating *V. grisco-vitellina* the type. He followed this in 1889 by leaving *V. grisco-vitellina* in the genus (adding two further species), and relegated *V. rufo-olivacea* to the genus *Lachnella* "Fries." He unquestionably selected a lectotype by this division.

If what we had agreed to call *Tapesina* need now be termed *Velutaria*, it is necessary to find a name for the species we were calling *Velutaria*. Unfortunately, this problem is by no means a simple one, and it may well be that no generic name is available for them. In his historical account of the genus *Velutaria*, Petrak (**1949**) makes no mention of *V. grisco-vitellina*, but rather merely states that *V. rufo-olivacea* is the type species. *Phacangium rubi* (Bäuml.) Sacc. is stated by Petrak to be a synonym of *V. rufo-olivacea*, and to be the "type" of *Phacangium* (Sacc.) Sacc. & Syd. 1902. It is similarly designated the type by Nannfeldt (**1932**). (The type, however, should be one of the original species included by Saccardo in *Cenangium* subgen. *Phacangium*.) In any case, this generic name is a later homonym of *Phacangium* Patouillard 1884. *Schweinitzia* Massee is also unavailable, as it is a later homonym of *Schweinitzia* Greville 1823.

The only generic name which might be legitimate appears to be *Phacangella* (Sacc.) Sacc. & Sacc. 1906. Petrak (**1949**) terms *P. aceris* (Hazsl.) Sacc. & Sacc. the "type" of the genus, again pre-

sumably because it was the first species listed in the genus (and likewise the first species listed in *Cenangella* subgen. *Phacangella* Sacc. 1889). Petrak suspects that *P. aceris* is also a synonym of *V. rufo-olivacea*. Should this lectotype prove to be the first designated and also to be a synonym of *V. rufo-olivacea*, a generic name would become available for that species and its allies.

A strict application of the rules would then result in the following (unfortunate) synonymy and typification:

VELUTARIA Fuckel, Symb. myc. 300. [1870.], *emend.* Sacc. Bot. Centralbl. **18**: 219. 1884.

SYNONYM: *Tapesina* Lambotte, Fl. myc. belg., suppl. 1, 305. 1887, *emend.* Holmel, Sitz.-ber. Akad. Wiss. Wien, Math.-nat. Kl., I, **132**: 110. 1923. [Lectotype: *Peziza ruborum* Cooke & Phil. (*Tapesina grisco-zitellina* (Fckl.) Holm. Ber. Deutsch. Bot. Ges. **37**: 108. 1919.)]

TYPE SPECIES: *Peziza grisco-zitellina* Fckl. (*Velutaria grisco-zitellina* (Fckl.) Fckl. Symb. myc. 300. [1870.]), designated by Saccardo (**1884**).

? PHAEANGELLA (Sacc.) Sacc. & Sacc. Syll. Fung. **18**: 128. 1906, *emend.* Petrak, Sydowia **3**: 199. 1949.

SYNONYMS: *Cenangella* subgen. *Phacangella* Sacc. Syll. Fung. **8**: 587. 1889. [Lectotype: *C. aceris* (Hazsl.) Sacc., selected by Petrak (**1949**).]

Cenangium subgen. *Phacangium* Sacc. Syll. Fung. **8**: 570. 1889. (Lectotype: ?)

Schweinitzia Massee, Brit. fungus-fl. **4**: 134. 1895. (Lectotype: ?) Not *Schweinitzia* Greville, Edinburgh Phil. Jour. **8**: 258. 1823.

Phacangium (Sacc.) Sacc. & Syd. Syll. Fung. **16**: 764. 1902. (Lectotype: ?) Not *Phacangium* Pat. Jour. de Bot., i, **8**: 155. 1894.

TYPE SPECIES: *Cenangium acceris* Hazsl. (a new combination in *Phacangella* would be necessary for *rufo-olivacea*), selected by Petrak (**1949**).

Both *Velutaria* and *Tapesina* as they are used in the literature are small genera, and it is questionable whether conservation should be utilized for such cases. When the rules result in the chaos exhibited above, however, it is necessary to consider whether the rules are wisely drawn. Many of the rules which have been proposed in the past have emphasized that usage is often a better guide than strict priority. Since the rules as now written include the priority concept, it would be necessary to conserve *Velutaria* in its current sense over *Velutaria* as emended by Saccardo, to avoid application of the rules. The case for conservation of small genera is tenuous, at best. Unless the rules of typification are revised, however, one must either conserve some such genera or submit to an upheaval of the nomenclature of such groups. I would personally prefer to see the rules revised to take into account predominant

usage, as in the case of *Velutaria*. If this is not feasible, conservation must be recommended.

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TWO TYPES OF SPORE GERMINATION IN SARCOSCYPHA COCCINEA (SCOP. EX FR.) LAMBOTTE

MARTIN A. ROSINSKI

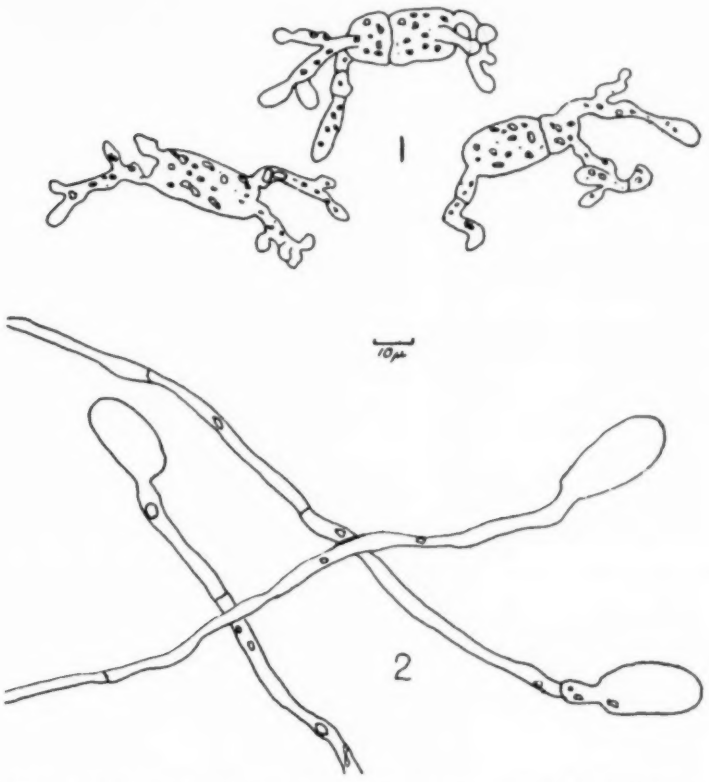
(WITH 6 FIGURES)

On April 19th, 1952, two collections of *Sarcoscypha coccinea*[1] were brought into the mycology laboratories at Cornell University. The collections were made independently from different local areas around Ithaca, New York. When these collections were placed side by side, it was obvious that there was a distinct color difference between them. In one collection, the hymenium was slightly on the orange side of scarlet (Séguy 167), while in the other collection the hymenium was a shade or two darker than scarlet (Séguy 61).

A variety, *S. coccinea* var. *jurana*, was described by Boudier (**1903**). He (**1906–1907**) later illustrated *S. coccinea* and *S. coccinea* var. *jurana*, showing a difference in the color of the hymenium and in the shape and size of the ascospores. In variety *jurana* he shows the hymenium with a brighter, more orange-red color, and the ascospores with flattened or blunt ends. In fact, in some cases the spores appear not only flattened, but indented at the ends. Ascospores from our collections which had been shot onto clean slides and onto agar showed a striking difference in morphology, strongly suggestive of the two forms illustrated by Boudier (FIGS. 3 and 5). However, the color of the hymenium in relation to the shape of the ascospores in our collections was the exact reverse of that indicated by Boudier. Perhaps color varies not with the variety but with the individual collection.

Spore measurements taken from the two collections (50 spores each) were as follows: 6.6–10.6 × 20–29 μ from the collection with blunt spores (presumably var. *jurana*), and 8–12 × 20–34.5 μ for the collection with tapered spores. Le Gal (**1941**) has shown that

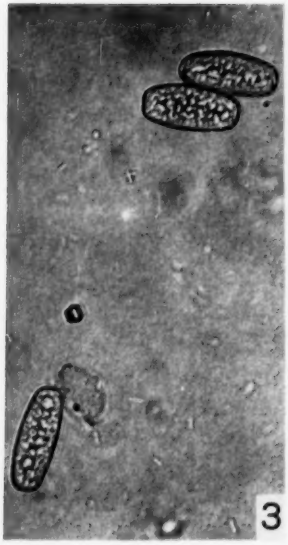
[1] The valid generic name for the scarlet-cups is *Sarcoscypha*, as pointed out by Korf (**1953**).

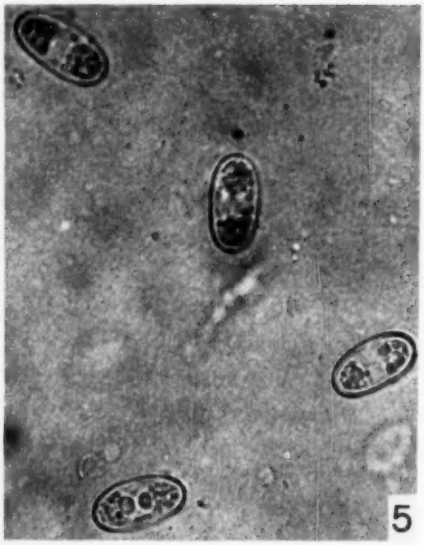


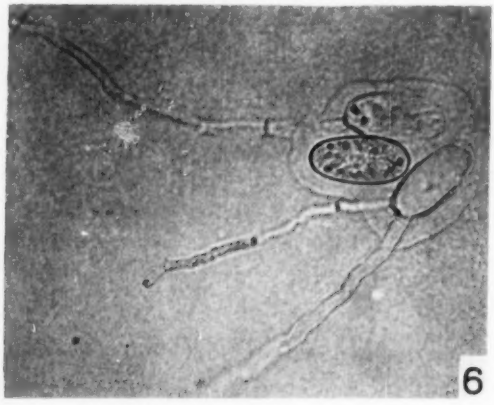
FIGS. 1–2. Spore germination in *Sarcoscypha coccinea* on water agar after 4 days. 1. *S. coccinea* var. *jurana*. 2. *S. coccinea* var. *coccinea*. Camera lucida drawings, at × 740, reduced in reproduction to × 500.

spore measurements vary considerably in this species, and the above figures are probably not significant. She also points out that some blunt spores may be found in variety *coccinea*[2] and that some tapered spores may be found in variety *jurana*. She concludes that the difference is not sufficiently constant to maintain a distinction. It is possible that the two types of spore germination to be de-

[2] According to the new rule established at the Stockholm meetings in 1950 (Lanjouw **1951**), the establishment of *S. coccinea* var. *jurana* Boud. automatically erected another variety, *S. coccinea* var. *coccinea*, which is to be cited without an author's name.







FIGS. 3-4. *S. coccinea* var. *jurana*. 3. Spores on water agar after 24 hrs. 4. After 4 days.

FIGS. 5-6. *S. coccinea* var. *coccinea*. 5. Spores on water agar after 24 hrs. 6. After 4 days. All photomicrographs reproduced at × 500.

scribed may lend support to Boudier's conclusion that there are two taxons involved.

To observe spore germination, portions of apothecia were inverted over petri dishes of water agar and of potato-dextrose agar.

The apothecia were allowed to shoot their spores onto the agar for a period of 24 hours. Ascospores were observed by cutting out blocks of the agar from areas where it could be seen that spore discharge had occurred. These blocks were placed on clean slides, and slide cultures were made according to the technique of Riddell (**1950**). Subsequent observations were made at 24 hour intervals.

Spores from both collections were observed to germinate on water agar within 48 hours. Little or no germination on potato-dextrose was observed. In the case of the collection with blunt spores, which we are calling *S. coccinea* (Scop. ex Fr.) Lambotte var. *jurana* Boud. (CUP *38758*), germination on water agar was observed to occur in the same fashion as that noted by Alexopoulos and Butler (**1949**). After swelling of the spores, from one to four short germ tubes were produced which soon gave rise to small buds or "conidia" as they were termed by the workers mentioned above. Camera lucida drawings (FIG. 1) and a photograph (FIG. 4) show this mode of germination. Following germination, the ascospore itself may become one or more septate.

On the same medium, a strikingly different spore germination was noted in the other collection, which we are calling *S. coccinea* (Scop. ex Fr.) Lambotte var. *coccinea* (CUP *38757*). After swelling of the ascospores, in most cases only one germ tube was produced from a single spore, and never more than two. Further, the germ tubes, instead of producing "conidia," merely increased in length, became multiseptate, and ultimately started to branch and form a limited mycelium. Camera lucida drawings (FIG. 2) and a photograph (FIG. 6) show this type of germination.

The significance of these results is not, as yet, known. They are results from only two isolated collections. Further work should prove whether or not the two types of germination which we observed are a fundamental difference between the two varieties.

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NOTES AND BRIEF ARTICLES

Closure for Culture Bottle

Aerobic micro-organisms that grow rapidly in surface culture often need a larger opening in the culture vessel, in proportion to the growth surface area, than micro-organisms that have a slow rate of growth. It is, therefore, frequently necessary to grow micro-organisms in wide-mouth bottles rather than narrow-mouth bottles to obtain greater interchange of air. A cotton plug could be used as a closure for the wide-mouth bottle, but it has well-known disadvantages. We have devised the bottle cap assembly shown in Fig. 1 as a substitute for the cotton plug. This assembly is made from a standard molded plastic cap, filter paper, a rubber band, and a prepared glass sleeve. The top of the plastic cap is cut out, so that the diameter of the opening is approximately equal to the diameter of the bottle mouth. Sufficient edge is left on the cap to give a good seal with the glass edge of the bottle. A snug fitting flat rubber band is placed on the outside of the cap to prevent the glass sleeve from slipping, and also act as a cushion to close crevices in the folded edge of the filter paper. The cap is screwed on the bottle and covered with filter paper and the glass sleeve is then placed over it.

For our assembly, Schleicher and Schuell [1] sharkskin filter paper was used. It has high wet strength, resistance to repeated steam sterilization and good air permeability. The number of filter papers used should be sufficient to give a tight fit between the glass sleeve and the cap; a minimum of three pieces is recommended. As many as twelve pieces can be used without significant effect on the growth of micro-organisms. It may be necessary to add more filter paper to some assemblies after repeated use to maintain a tight fit between the glass sleeve and the cap.

The glass sleeve is made long enough to reach the shoulder of

[1] Mention of brand names does not constitute endorsement or recommendation by the Department of Agriculture over similar brands not mentioned.

the bottle. This reduces the risk of entrance of contaminating particles, and also permits the assembly to rest on the table top while the cap is kept a safe distance above the table top. The top edge of the glass sleeve is turned in to decrease deposition of dust and foreign particles on the filter paper.

With this bottle closure, it is possible to inoculate one or a group of bottles very simply. The closure is unscrewed beforehand, but left on the top of the bottle. After addition of the inoculum, the

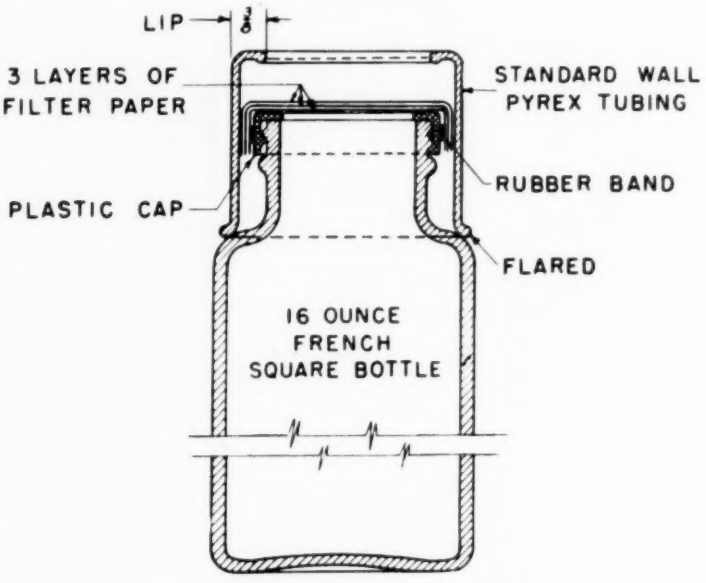

FIG. 1. Bottle with bottle cap assembly.

closure can again be placed on the bottle top, and screwed down later.

Use of this bottle closure has made it possible to obtain excellent spore crops from strains of *Aspergillus niger* and *Aspergillus oryzae* in a short time. Culture of these organisms on media supporting excellent growth in containers not having wide mouths closed with material of good air permeability showed suppressed spore formation.

Media cannot be stored for any length of time in wide-mouth bottles capped either with cotton plugs or the closure described herein without loss of water. We therefore explored the possibility of using the assembly with only a hole drilled in the top of the cap instead of having the entire top removed. This would restrict the loss of water and perhaps have little effect on the spore production of the micro-organism. A clearance between the top of the cap and the filter paper of at least $\frac{1}{16}$ of an inch was effected by a rubber washer $\frac{1}{8}$ inch wide and equal in diameter to the diameter of the cap. Sixteen-ounce French square bottles containing 50 mls. of whole potato media (7% solids) were inoculated with spores of *Aspergilus oryzae* NRRL *458* and incubated for 4 days at 30°C. Spore counts per bottle are shown below.

	Spores per bottle, billions
Closure having 1.5-inch hole (top of cap removed)	9.00
Closure having $\frac{1}{16}$-inch hole in cap	0.85
Closure having $\frac{1}{8}$-inch hole in cap	1.40
Closure having $\frac{1}{4}$-inch hole in cap	2.30
Closure having $\frac{1}{2}$-inch hole in cap	3.00

The spores in the bottles having holes less than 1.5 inches in diameter in the caps were smaller and lacked characteristic color.

The bottle closure described can be constructed easily and can be used over and over again without loss of its effectiveness. For quantity production, a solid plastic piece could be used in place of the glass sleeve and the plastic cap. The filter paper could then be cut to fit the inside of the cap opening, and held against it by a plastic ring or other suitable means. This arrangement would make it convenient to replace the filter paper. A closure of this type should be useful for many containers employed in culture work.—ELMER A. WEAVER, THEONE C. CORDON AND HARRY J. JOHNS, Eastern Regional Research Laboratory, Philadelphia 18, Pennsylvania.

A TECHNIQUE OF MOUNTING FUNGAL COLONIES FOR MUSEUM SPECIMENS

In the identification of many fungi in plate culture it is imperative to observe both sides of the colony. It is necessary to see the "reverse" or underside of the growth on agar to observe pigment pro-

duction and diffusion. This is true of many species, especially in the Fungi Imperfecti, as, for example, the Penicillia and Aspergilli. To have the spore-bearing apparatus in its normal arrangement with a three-dimensional configuration is desirable in most cases. Hence, to be able to study both sides of the mounted dried specimen under high-dry magnification with various adjustments of reflected or transmitted light calls for a suitable method of preservation and mounting.

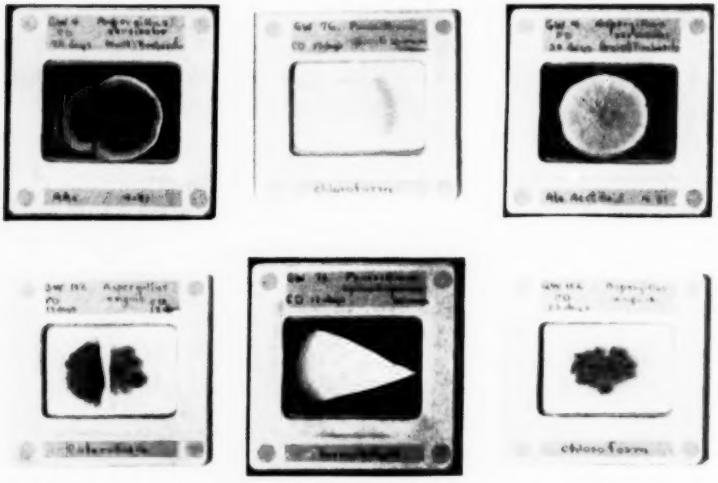

FIG. 1. Specimens showing several types of mountings for different fungi.

The method herein described makes use of 35 mm. mounting frames with one glass used in the frame. The Kodaslide Ready-mounts of the Eastman Kodak Company, the SVE Slide Binders of the Society for Visual Education, Inc., or the Automounts of E. Leitz, Inc. (all termed mounting frame here) serve the purpose. Whole colonies (6–8 days old, depending on the rate of growth) may be mounted in their entirety, or parts of colonies, preferably sectors, can be used where the young colony may not show certain details which are evident in an older one too broad to mount *in toto*. The specimens illustrated in FIG. 1 show several types of mountings of different fungi.

PROCEDURES

1. Growing the colony for mounting

Although any solid agar medium in a Petri dish can be used for growing the organism, one which differentiates certain characters may be more advantageous, such as Czapek-Dox modified agar for the Penicillia. Malt extract, potato dextrose, and corn meal agars are also satisfactory for growth but not always diagnostic. The last-named does not adhere well to the glass surface if it is very granular or has large pieces of the corn meal present.

2. Killing the organism

The use of non-viable mounts eliminates the possibility of contaminating the air with viable spores when studying the materials. Several milliliters of chloroform in the lid of the inverted dish overnight are sufficient to kill the mycelium and the spores. Formaldehyde also can be used as the killing agent, but the resulting product is not as satisfactory because of wetting and matting of the aerial mycelium and crystal formation on the surface. No growth could be obtained from transfers made from specimens treated with chloroform or formaldehyde, whereas material treated with ether or benzene remained viable. Also, some killing agents dissolve pigments from the cells followed by diffusion of color into the agar.

3. Preparation of colony for mounting

When the growing colony has reached a convenient size, a sector or the entire colony can be cut (after killing) from the rest of the agar plate with a large cork borer, a spatula, or an inverted shell vial. The fungus and the adhering agar are placed directly onto the glass slide and air dried. A convenient method to prevent dust accumulation is to place the slide and the mount in an empty Petri dish with the lid ajar. No adhesive, such as albumen fixative, is needed, since the agar acts as such on drying. The mount is dry in 24–48 hours, depending on the age and thickness of the agar, and the water content of the whole mount. When the colony is dried, the glass can be sealed between the two sides of the mounting frame and labeled.

4. Storage of the mounts

A thin cover slip or cover glass may be added over the colony by elevating it with two thin pieces of glass or thick mounting material as balsam at the four corners; however, to be able to examine the specimen under high-dry magnification it seems best not to put a cover above the fungus, which necessitates keeping the museum specimens separated in a "Unifile" slide case where they cannot rub against each other. The uncovered mounts can then be stored individually in any file for 35 mm. slides. Cellulose acetate envelopes are available which can be used to store a greater number of mounts. These may, however, flatten the aerial surface if the mount is excessively thick.

Some fungi with much cottony, floccose hyphae wet and pack tightly, so that these mounts are not as satisfactory as others. To date, mounts have been stored in darkness at room temperature (22–26° C.) for a period of 16 months with no evidence of breaking or fading. Spore colors, including blues and greens, which often fade in dried, unkilled mounts, have in these preparations retained their natural colors. MARLIN A. ESPENSHADE, Department of Botany, George Washington University, Washington, D. C.

DISPOSITION OF NOMINA GENERICA CONSERVANDA PROPOSITA FOR FUNGI

The Special Committee for Fungi adopted at the VII International Botanical Congress at Stockholm five resolutions concerning nomina generica conservanda proposita:

I. That every proposal for conservation not "accompanied by a detailed statement of the cases for and against . . . conservation" [Art. 21, Note 1, of the 3rd edition of the Rules; Art. 24, Note 1, of the new Code] nor having such a statement supplied by a later student, is to be rejected, to be reinstated as a subject for consideration by the Committee only after such a statement is supplied.

The list of proposals so rejected need not be set forth here. It may be that proposals with such statements have escaped the attention of the Committee; if so, these are subject to later action. It is here implied, however, that all proposals submitted before July 1,

1950, and not referred to in succeeding paragraphs are automatically rejected under this first resolution.

II. That every proposal for conservation of a name that would be retained without conservation against the nomina rejicienda proposita is to be rejected.

The following proposals are automatically rejected under this second resolution:

Agaricus vs. Psalliota & Pratella
Auricularia vs. Auricularia & Laschia
Calocera vs. Corynoides
Clitopilus vs. Pleuropus
Collybia vs. Gymnopus
Cordyceps vs. Cordiceps
Cystopus vs. Cystopus
Cyttaria vs. Cyttarium
Flammula vs. Flammula, Gymnopilus, Gymnocybe, Flammopsis, Ryssospora, & Visculus
Gomphus vs. Gomphora
Guepiniopsis vs. Guepinia
Guignardia vs. Carlia
Gymnosporangium vs. Puccinina, Aecidium, & Roestelia
Hericium vs. Hericium
Hexagona vs. Scenidium
Hydnum vs. Dentinum
Hydrocybe vs. Leucopus
Hymenochaete vs. Stereum
Hymenogramme vs. Aschersonia, Junghuhnia, & Laschia
Hypomyces vs. Hypolyssus
Marssonina vs. Marsonia & Marssonia
Melampsora vs. Uredo
Nidularia vs. Granularia
Octaviania vs. Octavianina
Olpidiopsis vs. Pleocystidium & Diplophysa
Omphalina vs. Perona
Panus vs. Rhipidium
Paxillus vs. Rhymovis & Ruthea
Peziza vs. Aleuria
Phallus vs. Ithyphallus
Phellinus vs. Poria
Pholiota vs. Derminus
Phoma vs. Sphaeropsis
Pisomyxa vs. Bryocladium
Plowrightia vs. Dothidella
Podoscypha vs. Craterella
Poria vs. Poria & Physisporus

Porodisculus vs. Enslinia
Pseudolpidium vs. Olpidiopsis
Pterula vs. Pterula
Puccinia vs. Puccinia & Puccinia
Secotium vs. Endoptychum
Sphaeropsis vs. Macroplodia
Stereum vs. Auricularia & Stereum
Telamonia vs. Raphanazon
Tomentella vs. Hypochnus
Tremella vs. Tremella & Gyraria
Trichosporium vs. Alytosporium
Venturia vs. Endostigme
Volvaria vs. Pseudofarinaceus
Xerocomus vs. Versipellis

III. That every proposal for conservation of a name that is a later homonym of a name in current use for a genus in another group is to be rejected; and that consultation is to be immediately undertaken with the appropriate Special Committees concerning all other names that are later homonyms of names in other groups.

The following proposals are automatically rejected under this third resolution:

Galera vs. Galera
Psathyra vs. Psathyra
Sphaerella vs. Sphaerella & Mycosphaerella

IV. That the following names proposed for conservation and about which there seems to be little difference of opinion are to be conserved, such action to be automatically rescinded if their present status is altered by action of the Stockholm Congress on the Rules:

[Note that this list is published in Int. Code Bot. Nomencl. pp. 76–78. 1952. The following corrections in that published list should be noted:

p. 76, right column, last line: for "1823" read "1822."
p. 77, right column, l. 19: after "1843" add "T.: idem."
p. 78, right column, l. 2: for "**1**: 6" read "**1**: 7."
p. 77, right column, l. 47, 48: for "Schw. ex Fr.," etc., read "Schw., Naturf. Ges. Leipzig Schr. **1**: 108. 1822."
p. 78, left column, l. 2, 3: for "Pers. ex Fr.," etc., read "Pers., Mycol. Eur. **1**: 141. 1821."

p. 78, left column, l. 4, 5 and l. 6, 7: for "Tode ex Fr.," etc., read "Pers. ex Hook., Fl. Scot. **2**: 9. 1821."

Of these, the first three are typographical errors, and the last three are results of the application of the new Art. 22 f. None have any nomenclatorial consequences.]

Nomina conservanda	Nomina rejicienda
Aleurodiscus Rab. ex Cooke, Grevillea **3**: 136. 1875.—T.: *Thelephora amorpha* (Pers.) Fr., El. Fung. **1**: 183. 1828.	*Cyphella* Fr., Syst. Mycol. **2**: 201. 1822.—T.: *C. digitalis* [A. & S.] ex Fr., l.c.
Calvatia Fr., Summ. Veg. Scand. 442. 1849.—T.: *Bovista craniiformis* Schw., Amer. Phil. Soc. Trans. II **4**: 256. 1832.	*Langermannia* Rostk. in Sturm, Deutschl. Fl. III **5** (18): 23. 1839.—T.: *Lycoperdon giganteum* Batsch ex Pers., Syn. Meth. Fung. 140. 1801. *Hippoperdon* Mont., Ann. Sci. Nat. Bot. II **17**: 121. 1842.—T.: *H. crucibulum* Mont., l.c.
Clavaria Vaill. ex Fr., Syst. Mycol. **1**: 465. 1821.—T.: *C. fragilis* Pers. ex Fr., op. cit. 484.	*Clavaria* Stackh., Ner. Brit. 2 ed. x. 1816.—T.: *Fucus clavatus* Lamour., Dissert. Esp. Fucus 22. 1805.
Daldinia Ces. & Not., Soc. Crittogam. Ital. Comm. **1**: 197. 1863.—T.: *Sphaeria concentrica* Pers. ex Hook., Fl. Scot. **2**: 4. 1821.	*Peripherostoma* Gray, Nat. Arr. **1**: 513. 1821.—T.: idem. *Stromatosphaeria* Grev., Fl. Edin. lxxiii. 1824.—T.: idem. *Hemisphaeria* Nees ex Klotzsch, Acad. Caes. Leop. Carol. Nat. Cur. Nova Acta **19**, suppl. 1: 241. 1843.—T.: idem.
Marasmius Fr., Fl. Scan. 339. 1835.—T.: *Agaricus rotula* Scop. ex Fr., Syst. Mycol. **1**: 136. 1821.	*Micromphale* Gray, Nat. Arr. **1**: 621. 1821.—T.: *M. venosum* [Pers.] ex Gray, op. cit. 622.
Melanogaster Corda in Sturm, Deutschl. Fl. III **3** (11): 1. 1831.—T.: *M. tuberiformis* Corda, l.c.	*Bullardia* Jungh., Linnaea **5**: 408. 1830.—T.: *B. inquinans* Jungh., l.c.

Nomina conservanda	Nomina rejicienda
Panus Fr., Epicr. 396. 1838.—T.: *Agaricus conchatus* Bull. ex Fr., Syst. Mycol. **1**: 181. 1821.	*Pleuropus* [Pers.] ex Gray, Nat. Arr. **1**: 615. 1821.—T.: *Pl. fornicatus* [Pers.] ex Gray, l.c.
Pleurotus (Fr.) Kummer, Führer Pilzk. 24. 1871.—T.: *Agaricus ostreatus* Jacq. ex Fr., Syst. Mycol. **1**: 182. 1821.	*Crepidopus* [Nees] ex Gray, Nat. Arr. **1**: 616. 1821.—T.: idem. *Resupinatus* [Nees] ex Gray, op. cit. 617.—T.: *Agaricus applicatus* Batsch ex Fr., op. cit. 192. *Pterophyllus* Lév., Ann. Sci. Nat. Bot. III **2**: 178. 1844.—T.: *Pt. Bovei* Lév., l.c. *Hohenbuchelia* Schulz., Zool.-Bot. Ges. Wien Verh. **16**: Abhandl. 45. 1866.—T.: *A. petaloides* Bull. ex Fr., op. cit. 183.
Septobasidium Pat., Jour. de Bot. **6**: 63. 1892.—T.: *S. velutinum* Pat., op. cit. 62.	*Gausapia* Fr., Syst. Orb. Veg. 302. 1825.—T.: *Thelephora pedicellata* Schw., Naturf. Ges. Leipzig Schr. **1**: 108. 1822. *Glenospora* Berk. & Desm., Hort. Soc. Lond. Jour. **4**: 255. 1849.—T.: *G. Curtisii* Berk. & Desm., l.c. *Campylobasidium* Lagerh. ex Ludw., Lehrb. nied. Kryptogam. 474. 1892.—T.: deest.
Stagonospora (Sacc.) Sacc., Syll. Fung. **3**: 445. 1884.—T.: *Hendersonia paludosa* Sacc. & Speg., Michelia **1**: 353. 1878.	*Hendersonia* Berk., Ann. Mag. Nat. Hist. I **6**: 430. 1841.—T.: *H. elegans* Berk., l.c.
Tomentella Pers. ex Pat., Hym. Eur. 154. 1887.—T.: *Thelephora ferruginea* Pers., Mycol. Eur. **1**: 141. 1822.	*Caldesiella* Sacc., Michelia **1**: 6. 1887.—T.: *C. italica* Sacc., op. cit. 7.
Tuberculariа Pers. ex Hook., Fl. Scot. **2**: 9. 1821.—T.: *T. vulgaris* Pers. ex Hook., Fl. Scot. **2**: 9. 1821.	*Tuberculariа* Wigg., Primit. Fl. Holsat. 87. 1780.—T.: *Lichen ericetorum* Scop., Fl. Carniol. 2 ed. **2**: 359. 1772.

Nomina conservanda	Nomina rejicienda
Uromyces (Link) Unger, Exanth. Pfl. 277. 1833.—T.: *Uredo appendiculata* Pers., Syn. Meth. Fung. 221, 222. 1801.	*Nigredo* Rouss., Fl. Calv. 47. 1806.—T.: *Uredo betae* Pers., op. cit. 220. *Cacomurus* [Link] ex Gray, Nat. Arr. **1**: 541. 1821.—T.: *U. appendiculata*, l.c. *Pucciniola* March., Bijdr. Nat. Wet. **4**: 47. 1829.—T.: *P. diadelphiae* March., op. cit. p. 48.

V. That as soon as the Rules as amended by the Stockholm Congress are available, all proposals for conservation not adopted or rejected under the foregoing paragraphs [I–IV] are to be referred to the members of the Committee for a vote, and that six months after the date of mailing of blank ballots the votes returned are to be recorded and the decisions of the Committee published.

By a written vote, completed in February, 1952, the Committee adopted the following resolution:

The assent of three-fifths of the members voting shall be necessary for the adoption of any proposal for conservation.

By the same written ballot the following proposals for conservation were adopted:

Nomina conservanda	Nomina rejicienda
Amanitopsis Roze, Soc. Bot. France Bul. **23**: 50. 1876.—T.: *Agaricus vaginatus* Bull. ex Fr., Syst. Mycol. **1**: 14. 1821.	*Vaginata* Nees ex Gray, Nat. Arr. **1**: 601. 1821.—T.: *V. livida* Gray, l.c.
Amphisphaeria Ces. & Not., Soc. Crittogam. Ital. Com. **1**: 223. 1863.—T.: *Sphaeropsis conica* Lév. in Demidoff, Voy. Russ. **2**: 112. 1842.	*Sphaeropsis* Lév., l.c.—T.: idem.
Aschersonia Mont., Ann. Sci. Nat. Bot. iii **10**: 121. 1848.—T.: *A. taitense* Mont., l.c. p. 122.	*Aschersonia* Endl., Gen. Pl. Suppl. **2**: 103. 1842.—T.: *Laschia crustacea* Jungh., Batav. Gen. Kunst. Wetensch. Verh. 17 Deel, III Stuck: 75. 1838.
Candida Berkh., De schimmelgesl. Monilia 41. 1923.—T.: *C. vulgaris* Berkh., l.c. p. 42.	*Syringospora* Quinq., Arch. Physiol. Norm. Path. **1**: 293. 1868.—T.: *Oidium albicans*

Nomina conservanda	Nomina rejicienda
	Rob., Hist. Nat. Veg. Paras. 502. 1853. *Parendomyces* Queyr. & Lar., Soc. Méd. Hóp. Paris Bull. Mem. iii **28**: 136. 1909.—T.: *P. albus* Queyr. & Lar., l.c. *Parasaccharomyces* Beurm. & Gong., Trib. Méd. **42**: 502. 1909 [? Nomen provisorium. Cf. op. cit. p. 518].—T.: deest; sed conf. pp. 521, 524, "*P. Sambergeri*" (nom. nud.). *Pseudomonilia* Geiger, Centralbl. Bakt. 134. 1910.—T.: *P. albomarginata* Geig., l.c. p. 135.
Coniothyrium Corda, Icon. Fung. **4**: 38. 1840.—T.: *C. Palmarum* Corda, l.c.	*Clisosporium* Fr., Syst. Mycol. **3**: 334. 1832.—T.: *Cl. lignorum* Fr., l.c. p. 335.
Conocybe Fayod, Ann. Sci. Nat. Bot. vii **9**: 357. 1889.—T.: *Agaricus tener* Schaeff. ex Fr., Syst. Mycol. **1**: 265. 1821.	*Raddetes* Karst., Hedwigia **26**: 112. 1887.—T.: *R. turkestanicus* Karst l.c. *Pholiotina* Fayod, l.c. p. 359.—T.: *A. blattarius* Fr., l.c. p. 246. *Pholiotella* Speg., Acad. Sci. Cordoba Bol. **11**: 412. 1889.—T.: *Pholiotella blattariopsis* Speg., l.c.
Gautieria Vitt., Monogr. Tuber. 25. 1831.—T.: *G. morchellaeformis* Vitt., l.c.	*Gautiera* Raf., Med. Fl. **1**: 202. 1828.—T.: *Gaultheria procumbens* L., Sp. Pl. **1**: 395. 1753.
Gyromitra Fr., Summ. Veg. Scand. 346. 1849.—T.: *Helvella esculenta* Pers., Mycol. Eur. **1**: 212. 1822.	*Gyrocephalus* Pers., Soc. Linn. Par. Mém. **3**: 77. 1824.—T.: *H. sinuosa* Brond., Soc. Linn. Paris Mem. **3**: [74]. 1824.
Mutinus Fr. Summ. Veg. Scand. 434. 1849.—T.: *Phallus caninus* Pers. Syn. Meth. Fung. 245. 1801.	*Aedycia* Raf., Jour. Bot. Soc. Bot. [Desvaux] **1**: 222. 1808.—T.: *A. rubra* Raf., l.c. *Ithyphallus* Gray, Nat. Arr. **1**:

Nomina conservanda	Nomina rejicienda
	675. 1821.—T.: *P. caninus*. *Cynophallus* (Fr.) Corda, Icon. Fung. **5**: 29. 1842.—T.: *P. caninus*.
Mycobonia Pat., Soc. Mycol. Fr. Bul. **10**: 76. 1894.—T.: *Peziza flava* Swartz ex Fr., Syst. Mycol. **2**: 161. 1822.	*Hirncola* Fr., Syst. Orb. Veg. 93. 1825.—T.: idem.
Panaeolus (Fr.) Quél., Soc. émul. Montbeliard Mém. ii **5**: 151. 1872.—T.: *Agaricus papilionaceus* Bull. ex Fr., Syst. Mycol. **1**: 301. 1821, emend. Fr., Epicr. 236. 1838.	*Coprinarius* (Fr.) Kummer, Führer Pilzk. 20. 1871.—T.: idem.
Phoma Sacc., Michelia **2**: 4. 1880.—T.: *P. herbarum* Westend. ex Sacc., Syll. Fung. **3**: 133. 1884.	*Phoma* Fr. ex Fr., Syst. Mycol. **2**: 546. 1823.—T.: *P. pustula* (Pers.) ex Fr., l.c. p. 547.
Pseudographis Nyl., Soc. Sci. Nat. Cherbourg Mém. **3**: 190. 1855.—T.: *Hysterium elatinum* (Ach.) Pers. ex Fr., Syst. Mycol. **2**: 584. 1823.	*Krempelhuberia* Massal., Geneac. Lichen. 34. 1854.—T.: *K. Cadubriae* Massal., l.c. p. 15.
Rhabdospora (Dur. & Mont. ex Sacc.) Sacc., Syll. Fung. **3**: 578. 1884.—T.: *Septoria Oleandri* Dur. & Mont., Expl. Sci. Alger. **1**: 593. 1849.	*Filaspora* Preuss, Linnaea **26**: 718. [1855].—T.: *F. perithecioformis* Preuss, l.c.
Rhipidium Cornu, Soc. Bot. Fr. Bul. **18**: 58. 1871.—T.: *R. interruptum* Cornu, l.c.	*Rhipidium* Wallr., Fl. Crypt. Germ. **2**: 742. 1833.—T.: *Agaricus stipticus* Bull. ex Fr., Syst. Mycol. 1: 188. 1821.
Urocystis Rabenh. ex Fuckel, Nass. Ver. Naturk. Jahrb. **23–24**: 41. [1870].—T.: *Erysibe occulta* Wallr., Fl. Crypt. Germ. **2**: 212. 1833.	*Polycystis* Lév., Ann. Sci. Nat. Bot. iii **5**: 269. 1846.—T.: *Caeoma pompholygodes* Schlecht., Linnaea **1**: 248. 1826. *Tuburcinia* Fr., Syst. Mycol. **3**: 439. 1832.—T.: *Rhizoctonia*. *Orobanches* Mérat, Nouv. Fl. Paris 2 ed. **1**: 135. 1821.

By the same written ballot the following proposals were rejected:

Acrotheca vs. Gomphinaria
Acrothecium vs. Cordana & Cacumisporium
Aposphaeria vs. Aposphaeria & Coniothyrium
Barya vs. Barya
Calodon vs. Hydnellum
Capnodium vs. Fumago, Apiosporium, & Polychaeton
Cladoderris vs. Cymatoderma
Clitopilus vs. Pleuropus
Coniosporium vs. Coniosporium
Coniothyrium vs. Clisosporium & Coniothyrium
Corticium vs. Phlebia, Corticium, Ricnophora, Athelia & Hyphoderma
Cortinarius vs. Cortinaria
Craterellus vs. Craterella
Dichaena vs. Heterographa
Dictyolus vs. Leptoglossum & Leptotus
Empusa vs. Empusa
Guepinia vs. Gyrocephalus & Phlogiotis
Guepinia vs. Guepinia
Hendersonia vs. Sporocadus & Hendersonia
Hirneola vs. Hirneola & Laschia
Hymenochaete vs. Hymenochaete
Hymenula vs. Hymenella
Hypochnus vs. Lyomyces
Hypolyssus vs. Caripia
Hypospila vs. Phoma
Lachnocladium vs. Eriocladus
Macowanites vs. Macowania & Hypochanum
Massaria vs. Splanchnonema
Mastomyces vs. Topospora
Monilia vs. Monilia
Monotospora vs. Monotospora
Montagnites vs. Montagnea
Mutinus vs. Aedycia
Mycoleptodon vs. Odontia
Mytilidion vs. Mytilinidion
Nummularia vs. Biscogniauxia, Nummularia, & Numularia
Ophiostoma vs. Endoconidiophora
Pactilia vs. Achitonium & Leucosporium
Peniophora vs. Corticium & Hyphoderma
Phleospora vs. Septoria
Phlyctidium vs. Phlyctidium
Pilacre vs. Phleogena
Polystictus vs. Coltricia & Strilia
Ramaria vs. Ramaria & Cladaria
Ramularia vs. Ramularia & Cylindrospora
Rhabdospora vs. Filaspora

Panellus vs. Rhipidium
Rhodophyllus vs. Acurtis, Entoloma, Leptonia, Nolanea, Eccilia, & Claudopus
Septoria vs. Septoria
Sordaria vs. Schizothecium, Enterobotyrum, Podospora, Malinvernia, & Pleurage
Spegazzinula vs. Dubitatio
Sphaeropsis vs. Macroplodia & Sphaeropsis
Stigmatea vs. Ascospora
Stigmea vs. Ascospora
Teichospora vs. Strickeria & Sphaeria
Thyrsidium vs. Cheirospora, Hyperomyxa, & Myriocephalum
Trematostoma vs. Clypeothecium
Tremellodon vs. Pseudohydnum & Hydnogloea
Trichosporium vs. Colletosporium & Alytosporium
Tromera vs. Biatorella & Sarea
Volvaria vs. Volvaria

The vote on the several proposals to conserve *Tricholoma* was inconclusive, and another vote is being taken.

It should be noted that the lists of rejected proposals are made up of abbreviated entries. In different proposals different types may have been considered for a single genus, and the nn. rejic. prop. may have been distributed among several separate proposals.

It should also be noted that the proposals approved by the written vote of the Committee are not thereby finally adopted. They are now submitted to the Advisory Board and the General Committee of Botanical Nomenclature (cf. Taxon **1**: 95) whose action will be reported in Taxon; if favorable action is received, they are tentatively adopted, and the conserved names may be retained "pending the decision of the next International Botanical Congress" (Int. Rules, 3 ed., Art. 22; Code, Art. 25). As a matter of practice, there seems no reason to anticipate the subsequent rejection of any of these proposals; all those known to conflict with names used in groups other than the Fungi have already been submitted (under the Committe's Resolution III) to the secretaries of the appropriate special committees.

Copies of new proposals for conservation of generic names of Fungi (with correct citation of names of genera and of types, and with arguments for and against conservation) should be sent to Prof. J. Lanjouw, Secretary, General Committee of Botanical Nomenclature, Lange Nieuwstraat 106, Utrecht, Netherlands, and

to the undersigned. To facilitate study and discussion before the 1954 Congress by those interested, authors are urged to publish such new proposals before September, 1953.—DONALD P. ROGERS, Secretary, Special Committee for Fungi, The New York Botanical Garden, Bronx Park, New York 58, N. Y., U. S. A.

SYDOWIA

Sydowia, the Journal of Taxonomic Mycology, established by Dr. Franz Petrak of Vienna, Austria, following the death of H. Sydow, to succeed the Annales Mycologici is now in its 7th volume. A large amount of valuable taxonomic material has been issued in these volumes, much of it by Dr. Petrak himself. As might be expected in these trying times of rapidly increasing costs, the journal is experiencing financial difficulties and it is suggested that additional subscriptions will afford effective help. Institutional subscriptions are particularly desired. The journal is published by Ferdinand Berger, Horn, n-ö, Austria, at 40 Swiss francs per volume.

REVIEWS

PRÉCIS DE MYCOLOGIE. MYCOLOGIE GÉNÉRALE. MYCOLOGIE HUMAINE ET ANIMALE. TECHNIQUES, by M. Langeron. Deuxième edition, revue et augmentée, by R. Vanbreuseghem. ix–703 pp., 461 figs. Masson & Cie., Paris, 1952. Price Fcs. 4800 unbound (about $13.75); fcs. 5400 bound (about $15.50).

The first edition of Langeron's text, published in 1945, met with wide recognition. Before his death, Dr. Langeron had made notes in preparation for a revised edition. So far as available, these were turned over to Dr. Vanbreuseghem and the preparation of the new edition was entrusted to him. The new work is greatly enlarged, more so than the increased number of pages would suggest, since the larger page of the new edition accommodates approximately half as much material again as did a page of the first edition.

The general form of the older work is retained. The first nine chapters, comprising over half of the volume, constitute Part I, General Mycology. The first chapter, in which Langeron argued for the inclusion of the fungi in the Protista, is retained with little change. This is followed by a review of the classification of the fungi and an extensive treatment of their morphology, spore dispersal and sexuality. Part II, Techniques, is arranged as a single long chapter, in which the valuable information on this subject in the first edition is repeated, with some revision, and expanded. Part III, Medical Mycology, is entirely new and is the work of Dr. Vanbreuseghem. In its eighteen chapters, the principal groups of diseases are treated in alphabetical order, beginning with those due to Actinomycetes and ending with the sporotrichoses. There is no general bibliography, but in the first two parts footnotes are used freely and in the third part each chapter has its special bibliography.

The book is beautifully printed and the illustrations are carefully selected and clearly reproduced. It deserves a place in every laboratory where fungi are seriously studied.—G. W. M.

MICOLOGIA. MORFOLOGIA, BIOLOGIA, EXPERIMENTACION, by Luis C. Verna and Federico J. Herrero. 740 pp., 286 figs. Editorial "El Ateneo," Buenos Aires. 1952. Price 120 pesos (about $8.50).

The attempt is made to cover, in a volume of convenient size, the entire field of mycology. The first six chapters deal concisely with the history of mycology, the systematic position of the fungi and fungal morphology, cytology, biology, chemical composition, sexuality and genetics. A long chapter on mycological techniques follows. About a third of the book, divided into five chapters, is devoted to classification. Two additional chapters provide rather full discussion of human pathogens and of the industrial applications of fungi. A glossary, bibliography and index complete the volume. Without neglect of theoretical foundations, the emphasis is practical. This is reflected in the full treatment given to certain important groups, notably to the yeasts, the Aspergilli and the Penicillia.

The book is marred by too many misprints and by the misspelling of the names of authors ("Taxter," "Buillard," "Dangear," "Pearson" [for Persoon], "Engler y Plant") and of scientific names ("Zoofage," "Olipdiopsis," "Tricophyton," "Licoperdum," to cite only a few). Few of these will cause confusion, but it is to be hoped that they may be corrected in future printings. Some of the figures, especially those reproducing photographs, could be vastly improved. Figure 64, p. 146, is misleading in showing only two nuclei as the result of meiosis, even though in some cases only two of the resulting four nuclei actually function.

This is the most comprehensive modern treatment of the fungi in Spanish which has come to my attention. The authors, both of whom are professors in the National University of Tucumán, are to be congratulated on their achievement. The book will be useful to all working mycologists but it is particularly recommended to graduate students in English-speaking countries, who will find in it not only much valuable information but an extremely helpful introduction to mycological discussion in a language in which the mycological literature is becoming increasingly important.—G. W. M.

MANUSCRIPT

Publication in MYCOLOGIA is ordinarily restricted to members of the Mycological Society of America. Exceptions to this regulation require a favorable vote by a majority of the Editorial Board. When a paper has two or more authors, the person submitting the paper is expected to be a member.

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